Spatial Signalling of

Met in Cancer

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DECLARATION OF AUTHORSHIP

I confirm that the work presented in this thesis is my own and the work of other persons has been properly cited and acknowledged.

ABSTRACT

Met, the receptor of Hepatocyte Growth Factor, is a receptor tyrosine kinase (RTK) overexpressed or mutated in cancer. RTKs have been increasingly recognised to signal post-endocytosis, possibly leading to unique consequences on cellular outcome due to the spatial and temporal activation of downstream signalling pathways. The objectives of my study were to investigate the role of Met "endosomal signalling" in cancer progression.

I found, using four human breast cell lines, that the requirement of Met endocytosis for Met signalling as well as Met trafficking significantly vary with the cells' aggressiveness, suggesting Met resides longer on endosomes in invasive cells. Furthermore Met endosomal localisation increases with the progression of breast cancer of human samples. Our study suggests that the endosomal location of Met is important in breast cancer progression.

I used a model of Wt and two Met oncogenic mutants, M1268T and D1246N, expressed in NIH3T3 fibroblasts. I determined some mechanisms regulating the constitutive endocytosis and defect in degradation of the mutants. I established that targeting these mechanisms could be used to reduce Met mutants' tumourigenicity. Thus impairing c-Cbl or Grb2 expression and/or binding to Met mutant or restoring Met mutant degradation through inhibiting the chaperone protein HSP90, greatly reduced the transforming capacities of the mutants *in vitro* and *in vivo*, including the D1246N that I show to be resistant to small molecule Met inhibitors.

I demonstrated that Met activation leads to Met and β1-integrin co-trafficking. Once on endosomes, β1-integrin is necessary to sustain Met-dependent ERK1/2 phosphorylation, cell migration and *in vivo* metastasis as well as Met dependent anchorage-independent growth and tumorigenesis *in vivo*. Importantly this role of β1-integrin is matrix-adhesion independent and suggests the existence of a novel mode of integrin signalling; “an endosomal inside-in signalling”.

Thus, inhibition of Met endosomal signalling may provide a potential therapeutic option in preventing the development and progression of Met driven cancers as well as overcoming drug resistance.

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LIST OF ABBREVIATIONS

17-AAG	17-allylamino-17-demethoxy-geldanamycin
17-DMAG	17-dimethylaminoethylamino-17-demethocygeldanamycin
ADAM10	A Disintegrin And Matalloproteinase domain containing protein
ADP	Adenosine Diphosphate
AHA1	Activator of HSP90 ATPase homologue 1
AP-2	Adaptor Protein 2
APAF1	Apoptotic Peptidase Activating Factor 1
Arf6	ADP-Ribosylation factor 6
ATP	Adenosine 5’ Triphosphate
BCCs	Breast Cancer Cells
BRCA1	Breast Cancer 1
BSA	Bovine Serum Albumin
CALM	Clathrin Assembly Lymphoid Myeloid leukemia
Cbl	Casitas B-lineage Lymphoma
CCV	Clathrin-Coated Vesicle
Cdc37	Cell-Division-Cycle 37 homologue
Cdc42	Cell-Division-Cycle 42 homologue
CHC	Clathrin Heavy Chain
CHIP	C-terminus of HSC70-interacting Protein
CIN85	Cbl-interacting protein of 85KDa
CLASP	Clathrin-Associated Sorting Protein
CRK	CT10 Regulator of Kinase
CrkL	Crk-like protein
CTF	C-Termnal Fragment

Cy	Cyanine
Dab2	Disabled homologue 2
DCIS	Ductal Carcinoma in Situ
DCS	Donor Calf Serum
DMSO	DiMethyl SulphOxide
DNA	Deoxyribo Nucleic Acid
DTT	Dithiothreitol
ECL	Enhanced ChemiLuminescence
EDTA	EthyleneDiamineTetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
EEA1	Early Endosome Antigen 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
Endo180	Endocytic receptor 180
Epsin1	Eps15-Interacting protein 1
Eps15	Epidermal growth factor receptor Substrate 15
ER	Estrogen receptor
ERK 1/2	Extracellular signal-Related Kinase 1 and 2
ESCRT	Endosomal Sorting Complexe Required for Transport
FACS	Fluorescence Activated Cell Sorting
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Gab1	Grb2-Associated Binder 1
GEF	Guanine nucleotide Exchange Factor

GGA3	Golgi-localised Gamma ear-containing Arf-binding protein 3
GIST	Gastrointestinal Stromal Tumour
GPCRs	G-protein Coupled Receptors
Grb2	Growth-factor-Receptor-Bound protein 2
GTPase	Guanosine Triphosphatase
HEK-293	Human Embryonic Kindney 293 cells
Her2	Human Epidermal growth factor Receptor 2
HGF	Hepatocyte Growth Factor
HGFA	HGF Activator
Hip1	Huntingtin Interacting Protein 1
Hop	HSP70/HSP90-Organising Protein
Hrs	HGF-Regulated tyrosine kinase Substrate
HSC-70	Heat Shock Cognate protein 70
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
IAA	IodoAcetAmide
IgG	Immunoglobulin G
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
IP	Immunoprecipitation
Lamp1	Lysosomal-Associated Membrane Protein 1
LCIS	Lobular Carcinoma In Situ
LRIG1	Leucine Rich Repeats and ImmunoGlobulin-like domain1
JNK	c-Jun N-terminal kinase
MEK	MAPK or ERK Kinase

MAPK	Mitogen-Activated Protein Kinase
MDCK	Madin-Darby Canine Kidney cells
MesNa	Mercaptoethanesulphonate
MMP	Matrix MetalloProteinase
MP1	MEK Partner 1
MVB	MultiVesicular Body
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NK1	N-terminal Kringle 1
NK2	N-terminal Kringle 2
NSF	N-ethylalemide-Sensitive Factor
NTF	N-Termnal Fragment
PBS	Phosphate Buffered Saline
PDGFR	Platelet-Derived Growth Factor Receptor
PI3K	PhosphatidylInositol 3-kinase
PLC	PhosphoLipase C
PKC	Protein Kinase C
PTP1B	Protein Tyrosine Phosphatase 1B
Rac	Ras-related C3 botulinum toxin substrate
Ras	Rat Sarcoma
RCP	Rab Coupling Protein
RhoA	Ras Homolog gene family, member A
ROCK	Rho-associated protein Kinase
RON	Receptor d'"Origine Nantaise"
RTKs	Receptor Tyrosine Kinases

SARA	Smad Anchor for Receptor internalisation
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel electrophoresis
SF	Scatter Factor
SH3	Src-Homology-3
Shc	SH2-domain-containing
Ship1	SH2-domain-containing Inositol-5-Phosphatase
Shp2	SH2-containing protein tyrosine Phosphatase 2
SNARES	Souluble N-ethylmaleimide-sensitive factor attachment protein receptor
Sos	Son Of Sevenless
Src	Sarcoma
STAM	Signal Transducing Adaptor Molecule
STAT3	Signal transducer and activator of transcription 3
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor β
Tiam1	T-cell lymphoma invasion and metastasis-inducing protein 1
TKIs	Tyrosine Kinase Inhibitors
TPR	Translocated Promoter Region
Trp53	Transformation related protein 53
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
VEC	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

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INTRODUCTION

I-Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) comprise a family of cell surface receptors possessing a domain with intrinsic enzymatic activity allowing the transfer of a phosphate from ATP to the hydroxyl groups of tyrosine[1]. Through the transfer of phosphates, signals can be passed on, via the receptor, to other proteins with tyrosine kinase activity[1]; thereby allowing RTKs to be effective signalling molecules.

There are 58 various different RTKs, which are separated into 20 subfamilies depending on their structure[2]. All RTKs contain an extracellular, often glycosylated, ligand-binding domain; a transmembrane domain, consisting of a single helix; a juxtamembrane domain; a catalytic domain and a C-terminal tail. The majority of RTKs are made up of a single polypeptide chain though both the Met and Insulin receptor subfamilies exist as dimers **(Figure 1)**.

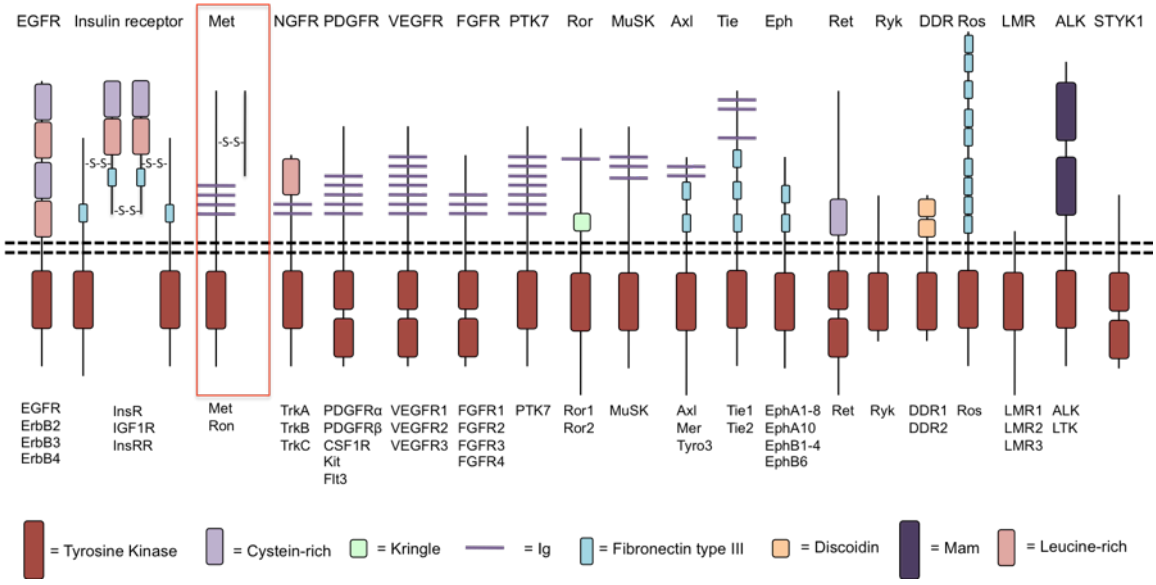


Figure 1: Receptor tyrosine kinases

The 20 subfamilies of RTKs are separated according to their structure.
All RTKs have an extracellular ligand binding domain, a transmembrane domain, a juxtamembrane domain, a catalytic domain and a C-terminal tail.

Based on Blume-Jensen. P & Hunter. T., Nature 2001[2] *and Lemmon. MA. & Schlessinger. J., Cell 2010*[3].

II-The RTK Met and its ligand HGF

1) Met

Met belongs to a small subfamily of receptor tyrosine kinases containing Met, Ron and avian Sea[4, 5] Met was first discovered as the TPR-MET oncogenic fusion protein in 1984 by Cooper et al.[6, 7]. A human osteogenic sarcoma cell line was treated with the carcinogenic compound N-Methyl-N-nitro-N-nitrosoguanidine, causing the chromosomal translocation of the TPR locus (Transocated Promoter Region) from chromosome 1 just in front of the Met gene[6, 7]. The result was the constitutively active TPR-MET oncogene. The carcinogenic compound that led to its discovery originally gave Met its name although it was later further named after its role in metastasis[8, 9]. A few years later Met was discovered to belong to the family of RTKs[10]. It is a proto-oncogene and is found expressed on the surface of epithelial and endothelial cells, where it binds specifically to its ligand, hepatocyte growth factor (HGF).

a- Gene

The Met gene is found on the long arm of chromosome 7 at band 7q31[11] (NCB1). It is greater than 120kb in length and contains 21 exons, divided up by 20 introns[12]. It shares many similarities with the Insulin receptor gene, which is reflected in a close homology of 44% between the two proteins[12]. Alternative splicing produces three isoforms of Met. The most abundant form of Met lacks 18 amino acids in the extracellular part of the receptor compared to a minor form of Met which, although it is able to undergo autophosphorylation, is unable to be cleaved proteolytically into the α and β

subunits [12, 13]. and it is unknown therefore whether or not it acts as a functional receptor *in vivo*[12]. Another isoform, $Met_{sm,}$ lacks 47 amino acids in the juxtamembrane domain that are important for Protein Kinase C phosphorylation, which is involved in downregulation of the receptor[12].

b-Structure

Met initially exists as an inactive precursor, consisting of a 170KDa single chain that undergoes proteolytic cleavage at a furin position to create the 190kDa mature form of Met[5, 14]. The mature form consists of an heterodimer of an α and a β chain that are linked via disulphide bonds[14]. The 45KDa α chain is extracellular, while the 145KDa β chain is both extracellular and intracellular since it crosses the plasma membrane. The receptor has a domain structure, with the extracellular part of the receptor containing a Sema domain, a Met Related Sequence, that is rich in cysteines, a region with numerous repeats containing many glycine and proline residues and four immunoglobulin-like regions[8, 15]. The extracellular region of the β chain, together with the α chain, forms the binding site for HGF[14, 16]. The intracellular part of the receptor contains a C-terminal region, a tyrosine kinase domain and a juxtamembrane region[14, 16]**(Figure 2)**.

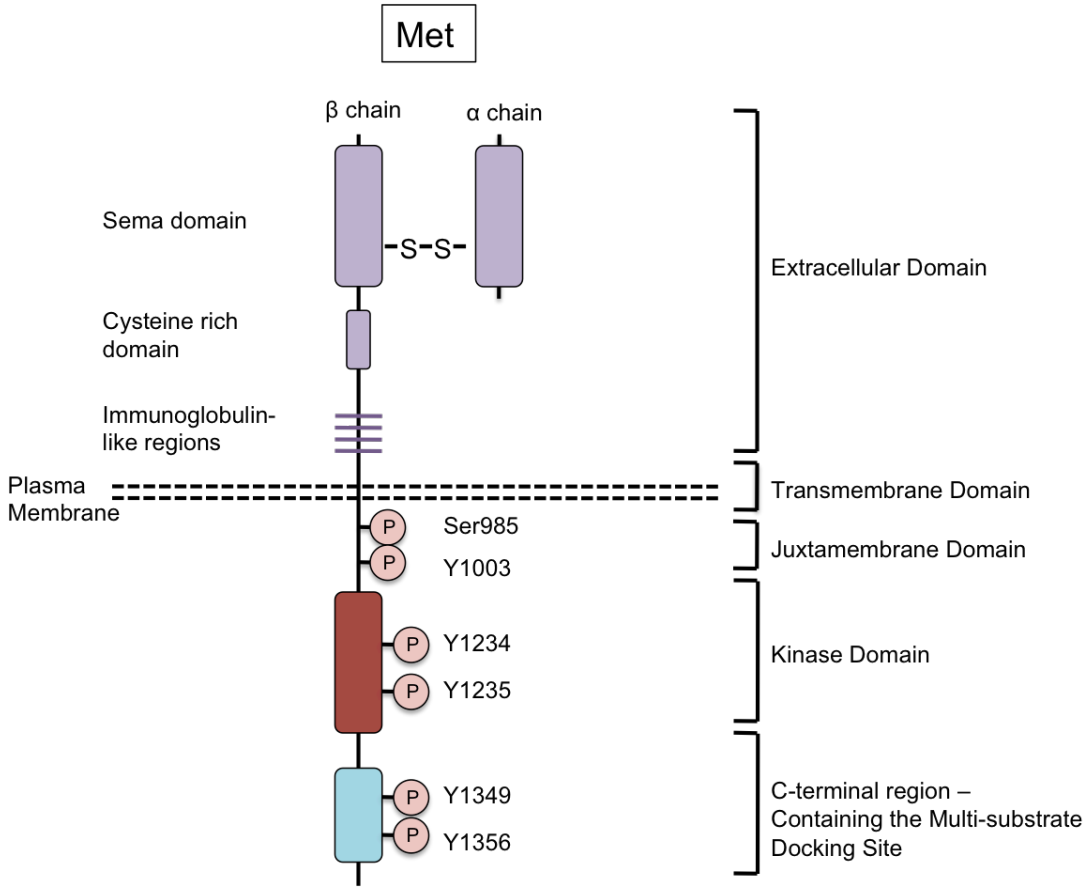


Figure 2: The structure of the Met receptor tyrosine kinase[16]

Met consists of an α and a β chain that are linked via disulphide bonds. The extracellular domain is made up of the α chain and part of the β chain, which forms the HGF binding site. The transmembrane domain crosses the plasma membrane, while the juxtamembrane domain contains the Ser975 and Y1003 residues that are involved in the negative regulation of Met. The kinase domain contains the Y1234 and Y1235 phosphorylation sites necessary for Met activation. Within the C-terminal region is a multi-substrate docking site containing the Y1349 and Y1356 phosphorylation sites required for downstream signalling.

Based on Trusolino. L. & Comoglio. P.M *Nature Reviews Cancer* 2002.[16]

2) Hepatocyte Growth Factor (HGF)

HGF is the only known ligand for Met[17] It is produced by mesenchymal cells, in close proximity to the epithelial/endothelial cells containing the Met receptor, as HGF has a restricted ability for diffusion, due to its high affinity for binding to heparin-sulphate proteoglycans[18]. HGF was first discovered in 1987 as both 'Scatter Factor' by Stoker et al.[19] and 'Hepatocyte Growth Factor' by Nakamura et al.[20]. Only a few years later, once HGF was cloned[21], did it become clear that these were in fact the same molecule[22-24].

a-Gene

The HGF gene is found on chromosome 7 at chromosomal bands 7q11.2-21[25], spanning approximately 70kb and consisting of 18 exons and 17 introns[25]. Alternative splicing leads to the production of three isoforms of HGF, including the full length form and two truncated forms: NK1 and NK2 ("N terminal Kringle", see below). Both of these truncated isoforms bind Met with high affinity but while the NK1 form can still initiate activation of Met downstream signalling, NK2 cannot and instead antagonises the effects of HGF[26].

b-Structure

HGF is part of the plasminogen family of proteins, however it has no proteolytic activity[16]. Like Met, HGF also has a domain structure, made up of an amino-terminal domain, four kringle domains and a serine proteinase homology domain[14, 15]. Initially, HGF is produced as a 92KDa single chain in

an inactive precursor form and this is then cleaved by serine proteinases that are often overexpressed in the tumour stroma[27] including hepsin, matripase, urokinase plasminogen activator (uPA), tissue-type plasminogen activator (tPA), coagulation factor X11 and the related molecule HGF activator (HGFA)[8, 28-30]. Inhibitors, including HGF activator inhibitor 1 and 2, can counteract the action of the HGF activating serine proteases[8]. The cleavage of HGF causes the formation of a bioactive heterodimer, consisting of a 69KDa α (the amino-terminal, with a hairpin loop, and four kringle domains) and a 34KDa β chain (the serine proteinase homology domain) that are linked via disulphide bonds[14, 15, 20]. The amino terminal domain and the kringle 1 domain form the binding site for the Met receptor[14]. It has been suggested that the dimer binds to two molecules of Met[31] **(Figure 3)**.

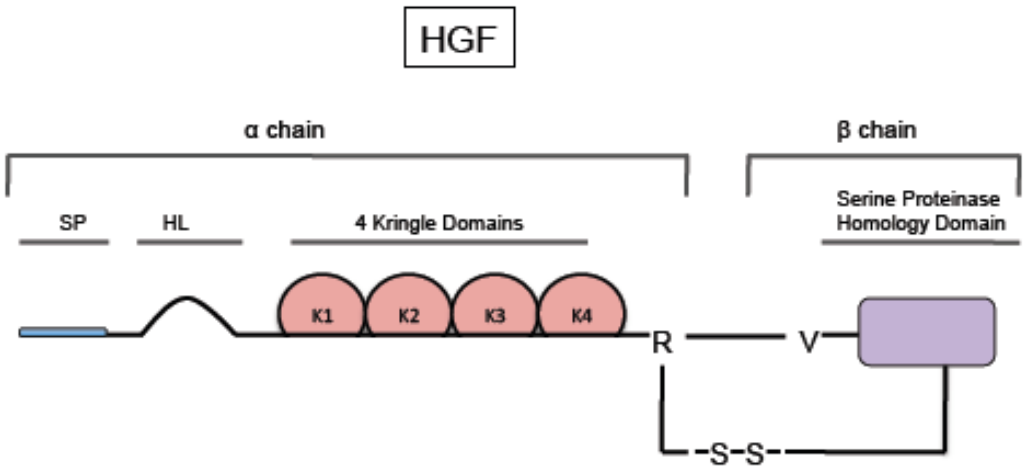


Figure 3: The structure of Hepatocyte Growth Factor[16]

HGF is produced as a single chain inactive precursor. Cleavage by serine proteases at a site between an arginine (R) and a valine (V), produces the mature form consisting of an α and β chain linked by disulphide bonds. HGF has a domain structure. The 69KDa α chain consists of an amino-terminal domain, within which is a single peptide (SP) and a hairpin loop (HL), and four kringle domains (K1-4). The 34KDa β chain contains a serine proteinase homology domain.

Based on Trusolino. L. & Comoglio. P.M Nature Reviews Cancer 2002[16]*.*

III- Met signalling

1) Met activation

Binding of HGF to the Met receptor leads to the stable dimerisation of two molecules of Met. This enables transautophosphorylation of the tyrosine kinase domain at tyrosine residues Y1234 and Y1235[15], followed by transphosphorylation of the two tyrosine residues Y1349 and Y1356[14, 15] in the C-terminal region **(Figure 2)**[27]. This forms a multisubstrate docking site, which is unique to members of the Met subfamily[32] and essential for Met signalling. It enables Met to bind to multiple substrates and activate a variety of signalling pathways either through direct interaction with signalling molecules or through adaptors[27, 32]. This site is unique to Met as most RTKs have several tyrosines located in their intracellular domain each of which is responsible for binding to a specific signalling molecule.

2) Adaptors

There are two major adaptor molecules that are essential for Met signalling; Grb2 (Growth Factor Receptor Bound Protein 2) and Gab1 (Grb2 Associated Binder 1). Gab1 binds to activated Met on either Y1349 or Y1356 through its specific 'Met binding site' consisting of 13 amino acids, leading to a strong interaction between Met and Gab1[33]. Gab1 contains multiple tyrosine sites that, when phosphorylated, mediate the additional binding of proteins including Phosphatidylinositol 3-kinase (PI3K), CT10 Regulator of Kinase (CRK), Phospholipase Cγ (PLCγ), SH2-domain-containing protein tyrosine phosphatase 2 (Shp2) and p120 Ras-GTPase-activating protein (p120-Ras-

GAP)[27, 33]. Gab1 can also bind to Met indirectly through the adaptor Grb2[34, 35], which binds to Met at Y1356/35[34], through an SH2 binding site[36]. Thus two molecules of Gab1 can bind, one directly and one indirectly, to Met simultaneously. It is worth noting that the docking site of a single Met molecule can only bind one substrate at a time, although in a Met dimer Gab1 can bind to one Met monomer at Y1349 and Grb2 at Y1356 on the other monomer simultaneously[14].

3) Signalling pathways

Various signalling molecules are recruited to activated Met either directly or through the adaptor proteins I have just described. Src, SH2-domain containing (SHC), CRK-like protein (CRKL), PI3K, Signal transducer and activator of transcription 3 (Stat3), PLC-γ, and Shp2 are all capable of binding to Met directly through their SH2 domain, with PI3K, PLC-γ, and Shp2 binding to Met through Y1356[27, 36].

In endothelial cells, activation of Shp2 via Gab1 has been shown to be important for branching morphogenesis and activation of the MAPK pathway[33], while activation of SOS (Son of Sevenless) through Grb2 leads to the activation of Ras[27, 37], which will lead consequently to activation of both the MAPK and PI3K pathways as well as activation of Rac1 and FAK, both of which are important regulators of migration and tubulogenesis[38]. Activation of phosphatidylinositol 3-kinase (PI3K), by either direct binding to Met or through Ras or Gab1, will lead to the activation of AKT which is involved in cell survival, cell-cycle progression, cell growth and cell metabolism[27, 39].

Upregulation of the MAPK pathway leads to the activation of ERK1/2, which is involved in cell proliferation, survival, differentiation, apoptosis, motility and metabolism[27, 40]. The transcription factor STAT3 plays an important role in Met-dependent, anchorage-independent growth and tumourigenesis[41], while the NF-κB transcription factors are also activated in response to Met activation[27]. Met signalling also has been linked to the JNK (c-Jun N-terminal kinase) through Gab1-Crk[42]**(Figure 3)**. Thus Met can activate a wide variety of signalling pathways and have multiple effects on cell function.

4) Negative regulation of Met

Due to the wide variety of cellular processes that are initiated following Met signalling, it is important that it is tightly controlled.

Down-regulation of Met signalling can be controlled by its degradation (see part IV-5).

Alternatively, in the juxtamembrane region there is a serine residue, serine 985, which gets phosphorylated by either PKC (Protein Kinase C) or Ca2+-dependent kinases when intracellular calcium levels increase upon Met activation[43]. The phosphorylation of this site reduces the tyrosine kinase activity of Met[44], acting as a negative feedback loop to help prevent continued uncontrolled Met signalling.

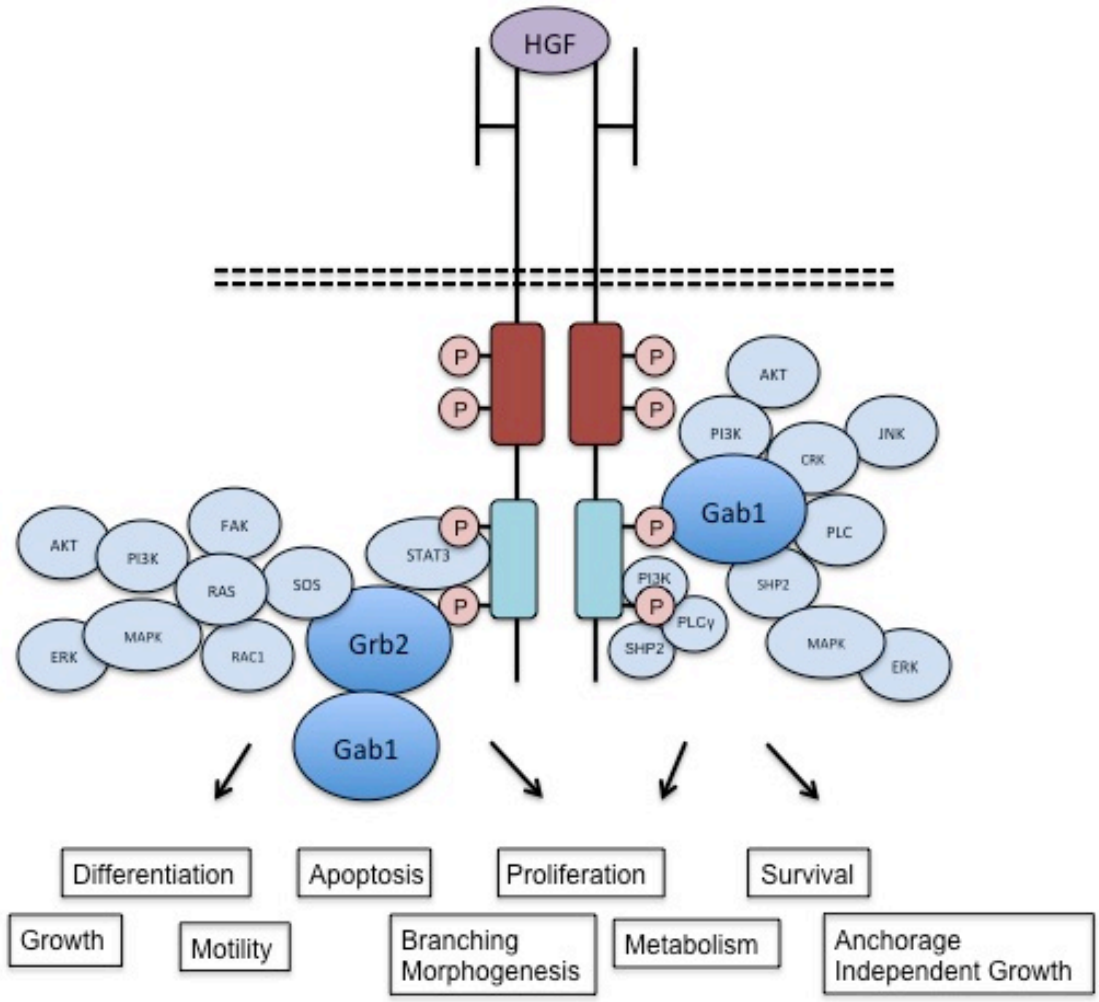


Figure 4: Signalling pathways initiated by Met signalling

Activation of Met upon HGF binding leads to the activation of multiple signalling moecules either through their direct binding to Met or by indirect binding though the adaptors Gab1 and Grb2. This leads to the activation of many signalling pathways that, in turn, induce the various cellular processes outlined above.

Based on Trusolino. L. & Comoglio. P.M Nature Reviews Cancer 2002[16].

5) Biological functions

The signalling pathways initiated by Met activation mediate the survival, motility and proliferation of cells. When responding cells are endothelial cells this leads to angiogenesis and 3D tubule formation, which is necessary for the development of many organs[14, 45]. *In vitro*, HGF stimulates the scattering and invasion of cells as well as the creation of 'branched tubules'[14].

Expression of both Met and HGF are necessary during foetal development. Mice lacking either Met or HGF expression die *in utero*[46-48]. Met signalling controls the proliferation and survival of placental trophoblasts[48]. It is also involved in liver development[46] and in promoting the release and migration of the progenitor cells that form various muscle groups[47].

In adults, expression of HGF and Met also often is increased in injured tissues[14]. Plasma levels of HGF are increased following liver[49] and heart injury[50] and Met expression in keratinocytes has been shown to be required for wound healing[51]. Furthermore, Met signalling is also involved in angiogenesis. Met is expressed on endothelial cells and HGF has been demonstrated to induce endothelial cell proliferation and migration *in vitro* as well as angiogenesis *in vivo*[52, 53]. Recombinant HGF treatment can increase VEGF (Vascular endothelial growth factor) production in keratinocytes as well as in cancer cells[54, 55]. The Met inhibitor PHA-665752 has been shown to reduce angiogenesis by both reducing VEGF production and by increasing production of thrombospondin-1, an angiogenesis inhibitor[56].

Angiogenesis is one of the six hallmarks of cancer originally proposed by Douglas Hanahan and Robert Weinberg in 2000[57]. These hallmarks are biological characteristics acquired by tumour cells during cancer formation; sustaining proliferative signalling, resisting cell death, evading growth suppressors, enabling replicative immortality, activating invasion and metastasis and finally inducing angiogenesis[57]. Met has been shown to be involved in many of these processes[8]. Interestingly, loss of pericytes, which are cells that surround and provide structural support to blood vessels, in breast cancer associated blood vessels, has been associated with an increase in hypoxia and increased Met activation and expression, which in turn correlates with reduced overall patient survival[58]. Deregulated Met signalling has also been shown to play a large role in cancer formation and progression through the activation of the many cellular processes it can initiate; an activity which I will describe in more detail later.

IV- Met endocytic trafficking

1) RTK endocytosis

Following receptor activation and dimerisation, RTKs undergo endocytosis in order that they can ultimately be degraded. There are various means of endocytosis, of which the most well characterised is the clathrin dependent pathway[59]. Alternative pathways include macropinocytosis and the lipid-raft dependent pathway, a subset of which is termed the caveolin pathway[59, 60]. RTKs mostly internalise by the clathrin or the caveolin pathway.

Clathrin dependent endocytosis relies on many adaptor proteins, the majority of which bind clathrin, the plasma membrane, the cargo as well as other adaptors with each playing a role in the formation of clathrin-coated pits[61]. These include the multimeric adaptor protein AP-2[60, 61] and the monomeric adaptors (clathrin-associated sorting proteins (CLASPs)) including CALM/AP180, Epsin1, Eps15, Dab2 and Hip1[61]. Together these complexes allow the formation of the clathrin coat in a pit-like structure around the cargo within the plasma membrane. Once formed, the GTPase dynamin, assembles and encircles itself around the top of the pits, allowing the clathrin coated vesicle (CCV) to break away[60, 61] **(Figure 4).**

2) Met endocytosis

Studies in HeLa cells have shown that internalisation of Met occurs via clathrin and dynamin mediated endocytosis, since knock-down of clathrin or

presence of a dominant negative version of dynamin prevents Met endocytosis[62, 63].

In order to initiate internalisation of the Met receptor, the E3 ubiquitin ligase c-Cbl (Casitas B-lineage Lymphoma) is required, which has two functions. Firstly, the ubiquitin ligase activity of c-Cbl is required when c-Cbl binds indirectly to Met via the SH3 domain of Grb2, which binds to Met at the phosphorylated Y1356 site[64-66]. In addition to the role of c-Cbl as a ubiquitin ligase it also acts as scaffold protein that is required for Met endocytosis. C-Cbl links Met to endophilins via the adaptor CIN85 (Cbl-interacting protein of 85 kDa)[67]. Endophilins are involved in invagination of the plasma membrane[68], which is one of the first steps in the initiation of clathrin dependent endocytosis. The direct binding of c-Cbl to Met and its ubiquitin ligase activity are also required for Met degradation and will be discussed in a later section. Clathrin adaptors regulating Met endocytosis are largely unknown and include so far AP180[62] and Eps15[69].

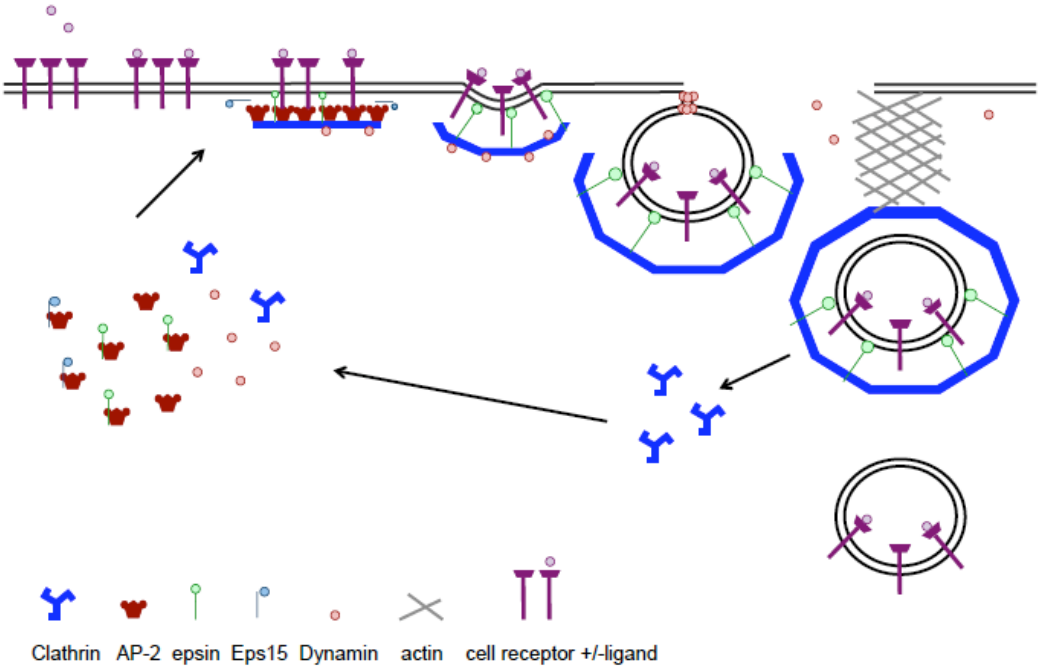


Figure 5: Clathrin mediated internalisation

Clathrin mediated endocytosis of cell surface receptors requires adaptor proteins, such as AP-2, epsin and Eps15, which bind to clathrin, the plasma membrane and the cargo. This leads to the formation of clathrin-coated pits around the cargo, which forms a clathrin-coated vesicle that eventually breaks away from the plasma membrane through the GTPase Dynamin.

Based on Rappoport. J.Z., Simon. S.M. & Benmerah. A. Traffic, 2004[70]

3) RTK trafficking

Once the clathrin coated vesicles have formed, clathrin is removed from the vesicles and the vesicles fuse with early endosomes in a process involving the GTPase Rab5[71]. The Rab5 effector EEA1(Early endosome antigen 1) is required for endosome docking and membrane fusion and as a consequence EEA1 is used as a marker of early endosomes[72]. SNARES (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) are present on vesicle membranes; they form tetrahelical complexes with each other resulting in membrane fusion[71].

Once in the early endosomal compartment, the vesicles can be sorted for degradation or they can be recycled back to the plasma membrane with their cargo, allowing RTKs to be stimulated by their ligand once again. This 'sorting' is controlled by Rab GTPases. Rab4 directs vesicles to a fast recycling route, whereas Rab8 and Rab11 direct them to a slow recycling route[71, 73]. Rab7 sorts vesicles to lysosomes for degradation [71, 73] **(Figure 6)**. Hrs (hepatocyte-growth-factor-regulated tyrosine kinase Substrate) is located on early endosomes[74, 75] and is involved in directing receptors to the MVB pathway for degradation[75, 76]. Hrs has been shown to sort ubiquitinated proteins into clathrin-coated microdomains of early endosomes through its ability to bind ubiquitin, phosphatidylinositol (3)-phosphate (an endosomal lipid) and clathrin[75-77]. Furthermore, Hrs is found in a complex with another ubiquitin binding protein STAM (Signal Transducing Adaptor Molecule), which together form the ESCRT-0 (Endosomal Sorting Complex Required for Transport-0) complex. ESCRT-0 is required for the recruitment of ESCRT-I,

which in turn recruits ESCRT-II and accordingly ESCRT-III and the consequent guidance of cargo into multivesicular bodies (MVB's) prior to lysosomal degradation[78-80].

It has been shown that different methods of internalisation can target RTKs for different fates. Sigismund et al. demonstrated that the dose of EGF was able to determine the route of internalisation and the consequent fate of EGFR[81]. A low dose of EGF leads to EGFR being internalised by clathrin-mediated endocytosis after which the majority of it is sorted for recycling back to the plasma membrane. Meanwhile a high dose of EGF causes EGFR to be internalised by a clathrin independent mechanism after which it is targeted for degradation[81]. Therefore, it is possible for the route of trafficking to be determined by the route of internalisation, and for the route of internalisation to be determined by levels of receptor ligand.

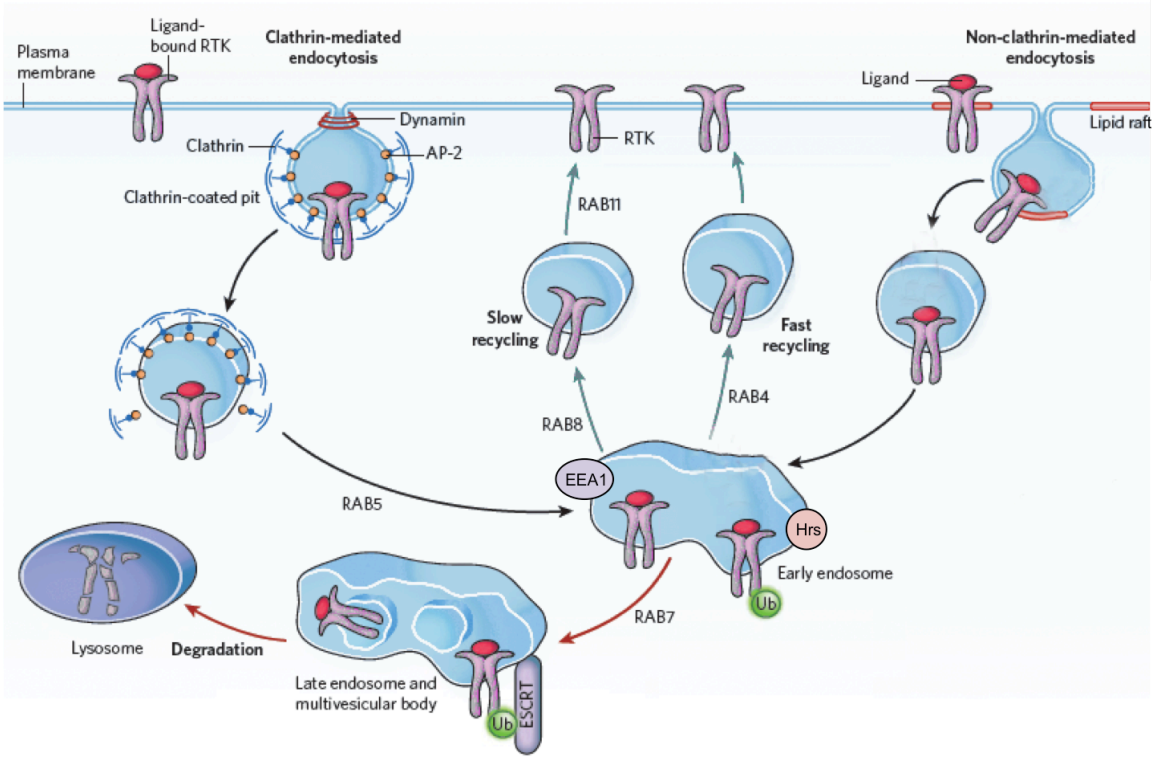


Figure 6: RTK trafficking

RTKs mostly internalise by the clathrin or the caveolin pathway. Once vesicles have been internalised they fuse with early endosomes in a process involving the GTPase Rab5[71], from where they are sorted by other RabGTPases. Rab4 directs vesicles to a fast recycling route, whereas Rab8 and Rab11 direct them to a slow recycling route[71, 73]. Rab7 together with ESCRT complexes, directs vesicles to the MVB prior to trafficking to lysosomes for degradation [71, 73]

Adapted from Scita. G. & Di Fiore. P.P. *Nature,* 2010[73]

4) Met trafficking

Met trafficking from an early endosomal compartment to a perinuclear compartment is operated along microtubules and promoted by Protein Kinase C- α (PKC α)[62, 82] (Figure 8). The non-receptor tyrosine phosphatase PTP1B has been shown to associate with Met and to be an important phosphatase in controlling Met phosphorylation upon HGF stimulation[83]. However it also has an important function in regulating vesicle fusion and hence in allowing Met to reach a perinuclear location[84]. This is believed to be due to the role of PTP1B in dephosphorylating N-ethylalemide-sensitive factor (NSF), which is involved in breaking up the SNARE complex and allowing additional vesicle fusion events[84].

Recently, Abella et al. demonstrated that dorsal ruffles, which are apical membrane protrusions, form following HGF treatment and act as a signalling microdomain for the Met receptor in MDCK epithelial cells[85]. Following HGF treatment, Met is transcytosed in Rab4 positive endosomes from the endosome to Gab1 positive dorsal ruffles on the apical surface of cells. In this microdomain Met remains active and furthermore phospho-Gab1 and phospho-ERK 1/2 are also found to be present, suggesting that dorsal ruffles provide a location for Met to signal to its downstream partners[85]. From here, Met is internalised into Rab5 positive compartments, traffics to Hrs positive compartments in a perinuclear location and then gets degraded **(Figure 7)**[85].

Met recycling has been reported through a specific interaction with Golgi-localised gamma ear-containing Arf-binding protein 3(GGA3) within Rab4

positive endosomes and this interaction is aided by Arf6 and Crk[86]. The resultant increased recycling of Met leads to a decrease in Met degradation, causing a less sustained ERK phosphorylation and reduced migration upon HGF stimulation[86].

5) Met degradation and stability

RTKs usually are targeted for degradation by ubiquitination. Once RTKs reach late endosomes they can enter the multivesicular body, which will fuse with lysosomes leading to their degradation.

a) Met degradation

The trafficking of Met to a perinuclear location is not required for its degradation[82]. The actual mechanism by which Met is degraded is unclear, since Met degradation has been shown to occur both by proteosomal[82] and lysosomal mechanisms[63, 87].

Phosphorylation of the tyrosine site Y1003 in the juxtamembrane of Met enables direct binding of c-Cbl. Although this interaction between Y1003 and c-Cbl is not required for Met endocytosis, it is required for ubiquitination of Met by c-Cbl and the consequent targeting of Met for degradation through the phosphorylation of Hrs [82, 88 89]. In fact, the fusion of a monoubiquitin to a ubiquitination deficient Met-Y1003F mutant, that does not directly bind c-Cbl, is able to restore normal Met degradation[89].

Additionally, LRIG1 (Leucine-rich repeats and immunoglobulin-like domains 1) can interact with Met in HEK-293T cells and destabilise Met in an HGF and c-Cbl independent manner[90].

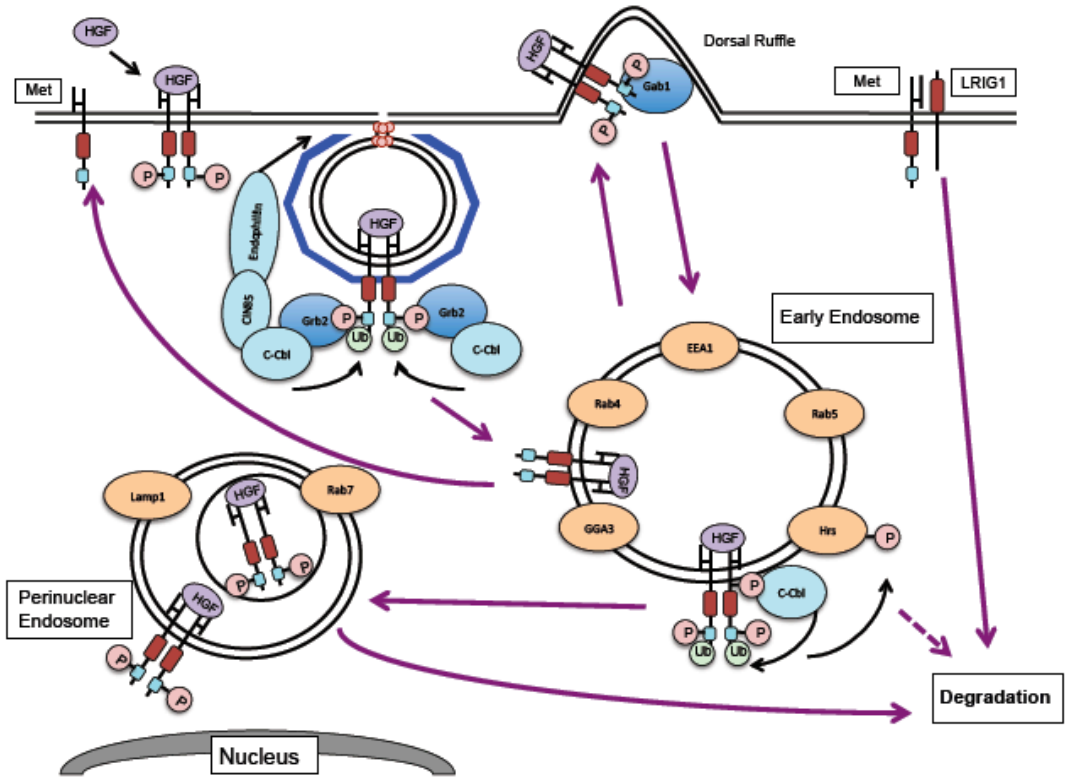


Figure 7: Met internalisation, trafficking and degradation

Met is internalised by clathrin dependent endocytosis upon HGF stimulation. c-Cbl is required for Met endocytosis by binding to Met via Grb2, leading to Met ubiquitination, or by acting as a scaffold between Met and CIN85-endophilin leading to membrane curvature. Met is recruited to the EEA1/Rab5/Rab4 positive early endosome where it remains phosphorylated. Binding of c-Cbl to Y1003 of Met, leads to Met ubiquitination and to the phosphorylation of Hrs, which leads to Met sorting for degradation. Some Met may recycle either to the plasma membrane through interaction with GGA3 or to Gab1 positive dorsal ruffles. From the early endosome, Met traffics to the Lamp1/Rab7 positive perinuclear endosome, where it is progressively targeted for degradation. LRIG1 may also interact with Met, leading to Met degradation.

b) Protection against degradation by HSP90

HSP90 (Heat-shock protein 90) is a molecular chaperone that has been demonstrated to stabilise Met and protect it from degradation[91]. I shall later outline in detail the role of HSP90 in cancer and in Met degradation, but I shall first describe HSP90 structure and function.

- HSP90

HSP90 is an evolutionarily conserved molecular chaperone that prevents misfolding, stabilises or ensures the correct activation of over 200 different proteins within cells[92, 93]. The chaperone Heat Shock Protein 70 (HSP70) is involved in recruiting the client protein to HSP90 with the help of co-chaperones. HSP90 is an ubiquitously expressed protein found mostly in the cytoplasm, which makes up between 1-2% of the total protein within a cell, however its expression increases under cellular stress and in cancer[94]. HSP90 often appears to be associated with mutated proteins involved in oncogenesis, possibly due to the high expression levels of the mutated protein but also due to their unstable conformations[94]. Mutated proteins found to be associated with HSP90 include v-src and p53[94]. It is possible for HSP90 to translocate to the nucleus or be secreted into the extracellular matrix where it has been shown to be involved in the maturation of MMP-2, which plays a role in tumour cell invasion[94, 95].

- HSP90 Structure

There are two cytosolic isoforms of HSP90; HSP90α and HSP90β (HSP86 and HSP84 respectively in mice)[96]. HSP90α is inducible, while HSP90β is

expressed constitutively[94, 97]. It is the HSP90α isoform that has been found to be upregulated in various tumours[94] and furthermore has been associated with a poor prognosis[98].

HSP90 is usually found as a constitutive homodimer, with dimerisation occurring at the carboxy-terminal end[97]. Each HSP90 monomer has three domains; an amino-terminal domain, which contains an ATP-binding site, a middle domain, which is involved in controlling ATP hydrolysis, and a C-terminal domain, where the dimerisation between two HSP90 monomers occurs[92].

- HSP90 Chaperone Cycle

HSP90 participates in a dynamic ATP-controlled chaperone cycle. As shown in Figure 8, upon ATP binding the homodimer undertakes a more closed conformation, with the N-termini of the monomers associating with each other and part of the N-terminus closing over the ATP-binding pocket.
Structural studies are still on-going and for the moment, although it is not clear exactly where the client proteins bind, several studies have suggested that they may bind in the middle domain[94, 99-101] .

Phosphorylation of HSP90 can occur at multiple tyrosine, serine and threonine sites and can have contradictory effects on HSP90 chaperone activity[93]. For example decreased phosphorylation of HSP90 at Ser226 and Ser255 increases the interaction between HSP90 and its client Apoptotic peptidase activating factor 1 (APAF1) in a subset of leukaemias[93, 102].

Meanwhile, the HSP90 client WEE1, which is involved in cell cycle progression, phosphorylates HSP90 at Y38 which increases the ability of HSP90 to chaperone cancer related proteins, including HER2, while simultaneously decreasing the binding ability of HSP90 inhibitors[93, 103].

- HSP90 Co-chaperones

There are at least 20 known co-chaperones that can either positively or negatively regulate HSP90 function in many different ways. They can be involved in the specific recruitment of client proteins to HSP90, the prevention or reduction in HSP90 ATP activity, control of the chaperone cycle or by engaging other chaperone molecules[92].

The co-chaperone Hop/STIP1 (HSP70/HSP90 Organising Protein) binds to the C-terminus of HSP90 and the C-terminus of HSP70 assembling an active complex, where the client protein is transferred from HSP70 to HSP90[100, 104, 105]. Alternatively, the co-chaperone CHIP (Carboxyl terminus of HSP70 Interacting Protein), an E3 ubiquitin ligase, is involved in ubiquitinating HSP70 clients and targeting them for degradation by the proteosome[106]. Often client proteins increase their assocation with CHIP when HSP90 inhibitors are bound promoting the rapid degradation of client proteins[94, 107].

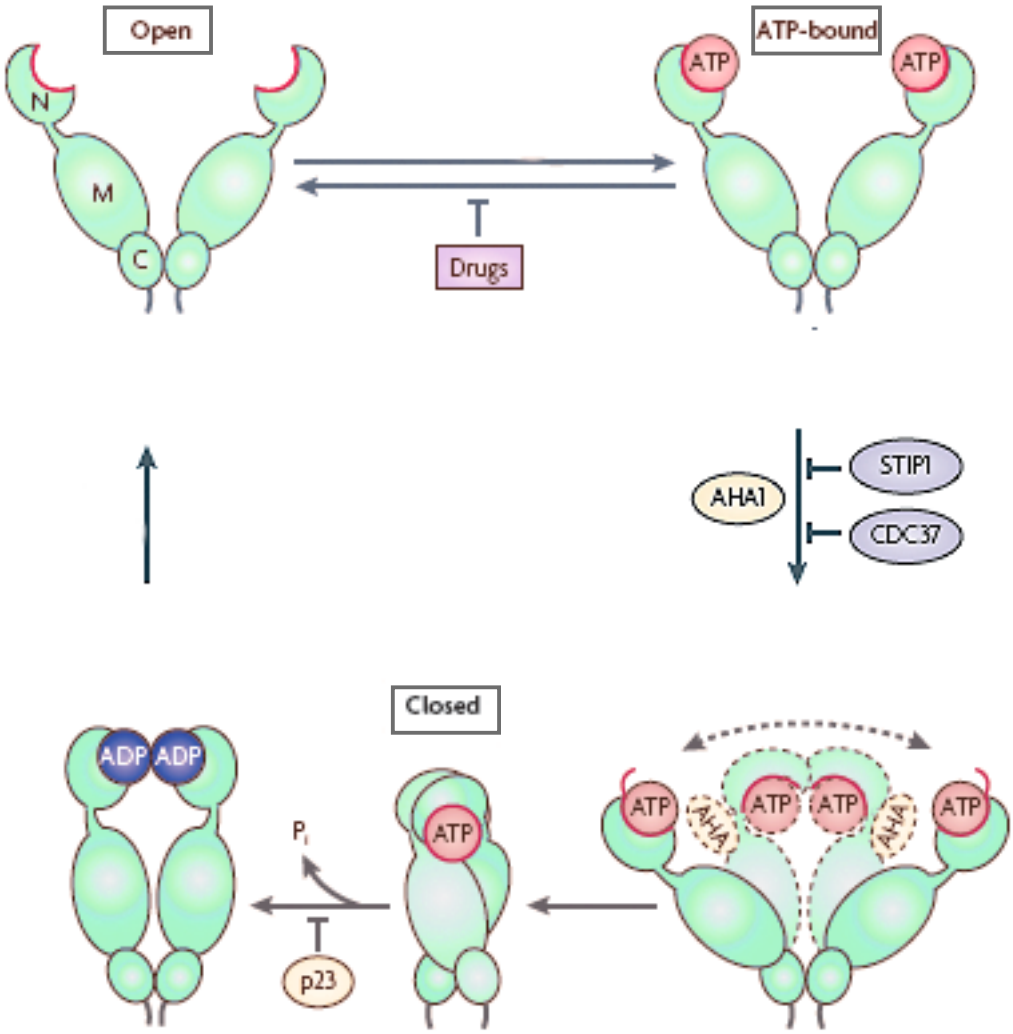


Figure 8: HSP90 Chaperone Cycle

ATP binds an HSP90 homodimer, causing a conformational change in the molecule, such that the N-termini of the monomers closely associate with each other. Co-chaperones can also play a role in altering the chaperone cycle, for example by changing ATP activity.

Adapted from Trepel 2010[93]

Other co-chaperones include CDC37 (cell-division-cycle 37 homologue), which is responsible for bringing kinases such as AKT[108] to the HSP90 complex, where it binds HSP90's N-terminus[94, 109] and the co-chaperone AHA1 (activator of HSP90 ATPase homologue 1), which increases the rate of ATP hydrolysis by HSP90 through encouraging the middle domain to associate with the N-terminus of HSP90[94]. Meanwhile, the co-chaperone p23 keeps HSP90 in the ATP hydrolysis state, promoting and maintaining HSP90's interaction with client proteins[92, 110-112].

- HSP90 in Cancer

In recent years, much work has focused on understanding the role of HSP90 in cancer cells, where it can protect a wide range of oncogenic proteins from being misfolded or degraded[93] (see Part 2). As a consequence there are a large number of pharmaceutical HSP90 inhibitors that have been produced and are undergoing, or are about to undergo, clinical trials in patients with advanced solid tumours including prostate, pancreatic, gastric and non-small cell lung cancer. HSP90 inhibitors are also being tested in haematological malignancies including in multiple myeloma patients for whom a few Phase 3 trials have recently been completed, but results are, as yet, unknown (*http://www.clinicalstrials.gov*).

1 – Inhibitors of HSP90

- *Inhibitors which bind to the N-terminal ATP-binding pocket*

The first pharmaceutical product that was discovered to have potential in inhibiting the action of HSP90 was the antibiotic Geldanamycin discovered in

1970[113, 114]. Since then, there have been several derivatives produced which have an improved toxicity profile and a higher specific affinity for HSP90.

A derivative of Geldanamycin, 17-allylamino-17-demethoxy-geldanamycin (17-AAG)(also known as KOS-953 or Tanespimycin), has similar activity to Geldanamycin but with lower toxicity issues[115] and became the first HSP90 inhibitor to enter clinical trials. It showed positive clinical benefits in several oncology trials including Her2 (Human Epidermal Growth Factor Receptor 2) driven breast cancer, however it had a high liver toxicity[93, 114]. Another geldanamycin derivative 17-dimethylaminoethylamino-17-demethocygeldanamycin (17-DMAG) had improved pharmacokinetics, however development appears to have stopped due to its high toxicity profile[93, 116].

Since, the discovery of these first HSP90 inhibitors, many more synthetic inhibitors have been developed or are currently under development by various pharmaceutical companies based on the structure of HSP90 using X-ray crystallography[114, 116]. The more recently produced inhibitors include STA-9090, the structure of which is not yet available, and the small molecule inhibitor SNX-5422, a prodrug that converts to SNX-2112, which have both shown promising results so far[114, 116].

- *Inhibitors which alter HSP90's interaction with its co-chaperones*

Novobiacin, a coumarin antibiotic, binds to HSP90 in its C-terminal domain and reduces its interactions with HSP70 and the co-chaperones p23 and

cdc37[94, 117-119]. While Novobiocin itself has a low affinity for HSP90, several derivatives have been produced that appear to have good anti-proliferative effects on several cancer cell lines[93 120-122].

The range of HSP90 inhibitors available demonstrates that HSP90 is a promising target in many cancers. There are currently 77 recruiting, ongoing or recently completed clinical trials (*http://www.clinicalstrials.gov*).

2 – HSP90 in cancer

- *The role of HSP90 in cancer cells*

HSP90, acts by helping cells respond to environmental stress. Cancer cells are continually under stress due to various factors including restricted nutrient and oxygen supply, genetic instability and assault by the host's immune system[114]. By protecting so many cellular proteins during such stress, HSP90 allows cancer cells to survive and proliferate causing some cancer cells to become dependent on HSP90[114, 123]. Importantly, HSP90 in tumour cells (and tumour tissue) has been found to be more highly associated with the co-chaperones p23 and Hop than HSP90 from normal cells[92, 124]. In addition HSP90 has also been found to have a higher ATPase activity in tumour cells[92, 124].

- *Inhibition of HSP90 in cancer cells*

Many clients of HSP90 inhibition are known oncogenes and HSP90 inhibition provides an alternative way to disrupt oncogene activity, particularly if such proteins are overexpressed or mutated, with the additional benefit that

multiple oncogenes can be inhibited simultaneously. A study by Kamal et al. demonstrated that 17-AAG binds to HSP90 from cancer cells with 100 fold greater affinity than HSP90 from normal cells[124].

As already mentioned, Her2 driven breast cancer has proven to be an HSP90 sensitive target in multiple studies, including in clinical trials[125-127]. Additionally, HSP90 inhibition has also been shown to be effective at inhibiting proliferation and inducing apoptosis in gastrointestinal stromal cancer cells (GIST) that were dependent on the Kit receptor tyrosine kinase, irrelevant of whether they were sensitive or insensitive to Imatinib (small molecule inhibitor of Bcr-Abl, Kit and PDGFRA)[128].

HSP90 inhibitors therefore seem to be highly effective in cancers driven by RTK overexpression; however studies have also shown them to effectively degrade mutant RTKs. Non-small cell lung cancer cells expressing the EGFR T790M mutant, that leads to resistance to the EGFR inhibitors Gefitinib and Erlotinib, remain sensitive to HSP90 inhibition[129]. Furthermore, a constitutively activated oncogenic mutant of the Ron receptor, Ron M1254T, was found to avoid c-cbl mediated downregulation but was more strongly associated with HSP90, HSP70 and CHIP than the WT receptor[130]. Moreover this mutant was more sensitive to treatment with the HSP90 inhibitors Geldanamycin or 17-AAG, partly due to a quicker dissociation of the HSP90-Ron M1254T complex[130].

- *Inhibition of HSP90 co-chaperones in cancer cells*

The expression of the chaperone HSP70 is often increased following treatment with HSP90 inhibitors, which may be one reason that HSP90 inhibitors have proved to be not as effective as first hoped in clinical trials[131]. Interestingly, inhibition of both HSP70 and HSP72 (the inducible isoform of HSP70) simultaneously, leads to degradation of HSP90 client proteins and apoptosis in a range of tumour cells, without affecting normal cells[131].

Some work has also been done on inhibition of HSP90 co-chaperones. For example, RNAi silencing of cdc37 has been shown to lead to degradation of HSP90 client proteins, specifically kinases and further sensitisation to HSP90 inhibitors without induction of a heat shock response that can occur upon inhibition of HSP90[132, 133].

3 – Met and HSP90

HSP90 has been shown to protect Met from degradation[91], particularly when it is acting as an oncogene. Consequently, Geldanamycins can induce Met downregulation and inhibit HGF induced migration and invasion of leiomyosarcoma or glioblastoma cell lines[91, 134]. Importantly, HSP90 inhibition has been demonstrated to provide long lasting Met inhibition. The Met amplified cell lines H1993 (non-small cell lung cancer) and MKN45 (gastric carcinoma), initially are sensitive to a Met small molecule inhibitor, however they quickly (after 48 hours) activate downstream signalling molecules via alternative mechanisms. Nevertheless, HSP90 inhibition in these cells

provides inhibition of both Met, and its downstream signalling molecules over a more extended period of time (up to 96 hours)[135].

The efficacy of HSP90 inhibition to downregulate Met has been confirmed with the recently produced HSP90 inhibitor; SNX-2112[136]. It has been shown to down-regulate Met and many other kinases effectively in Met-amplified cell lines; EBC1 (non-small cell lung cancer), GTL-16 and MKN-45 (gastric cancer) as well as a GTL-16 cell line made resistant to the small molecule Met inhibitor PHA-665752[136]. Furthermore, a decrease in tumour growth was observed *in vivo* when mice were treated with SNX-5422, the pro-drug of SNX-2112[136].

The data that I will present in my thesis are derived from investigation of the role of HSP90 inhibition in two naturally occurring Met mutations, one of which is naturally resistant to small molecule Met inhibitors.

c) Met Cleavage and Shedding.

Alternative methods of Met degradation, which occur in a ligand independent manner, are Met cleavage and Met shedding[66]. Met cleavage has been described to occur during apoptosis as a way to prevent potential survival signals[66]. Cleavage is carried out by caspases that cleave Met into a 100kDa membrane-bound fragment and a 40kDa C-terminal fragment containing the tyrosine domain, thereby making Met unable to undergo ligand-dependent activation[66].

Met shedding takes place to reduce accumulation of mature Met levels under basal conditions, independent of ligand stimulation, and it occurs through a process known as Presenilin-regulated intramembrane proteolyis (PS-RIP)[27, 66]. Initially Met Cleavage occurs by MMPs (Matrix Matalloproteinases), involving ADAM10 (A disintegrin and matalloproteinase domain containing protein), leads to the shedding of an N-terminal fragment (NTF), leaving behind a membrane bound C-terminal fragment (CTF)[66, 137-139]. This C-terminal fragment can either be directly degraded by the lysosome[140] or, consequently, can undergo cleavage by the γ-secretase complex (the catalytic subunit of which is Presenilin) to produce a C-terminal fragment that can traffic to the lysosome and a membrane-bound intracellular domain (ICD) that undergoes proteosomal degradation[27, 66, 137]. The expression of Trk-Met, lacking the juxtamembrane region, shown to be required for appropriate metalloproteinase cleavage of Met, led to a large increase in cell migration in MDCK cells[137]. The formation of Met fragments is increased following treatment of cells with the human Met antibody DN30[137]. Thus, Met cleavage and shedding provide another means for Met signalling to be discontinued.

V- Met endosomal signalling

1) The "Signalling endosome" concept

Despite the role of endocytosis in receptor degradation, receptors are still able to signal from endosomes[141]. It currently is becoming clear that this "endosomal signalling" of RTKs is important for regulation of cell functions since it provides temporal and spatial regulation of the signalling pathways, as well as enabling the direct transfer of signalling complexes to their intended target[141].

2) Endosomal signalling of receptors

Much of the current work on "endosomal signalling" has been carried out on EGFR, which was in fact the first RTK found to be present in endosomes with signalling partners[142]. The landmark study that founded the concept "endosomal signalling" was published in 1996 and showed that the Nerve Growth Factor (NGF) receptor can be found activated in endosomes together with its ligand[143]. This field has expanded rapidly since these first studies and has focused on trying to uncover the role of receptor localisation on cell signalling and function.

EGFR has been found to be able to initiate and stimulate signalling pathways from endosomes, including activation of Rac, ERK1/2 and AKT in BT20 breast cancer cells[144]. However, SHC and PLC-γ do not require endocytosis for their full activation and in fact are hyperphosphorylated if EGFR is maintained at the plasma membrane[145]. In endocytosis-defective cells, EGF

dependent cell proliferation is increased[145], while cell survival and apoptosis are decreased[144]. This suggests that different signalling pathways and consequently cell functions are initiated from different locations within a cell. Other RTKs have also been investigated. The same authors as above found that in the same way as EGFR, PDGFR is also capable of initiating signalling from endosomal compartments[146]. Endocytosis of the insulin receptor has been shown to be required for full SHC and ERK1/2 activation[147]. An association between Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2) with Vascular Endothelial Cadherin (VEC) inhibits VEGFR-2 endocytosis which, in turn, prevents cell proliferation[148].

Importantly, Jékely et al. demonstrated that RTK endocytosis is important for directional cell migration towards ligands during Drosophila development, making it the first study to suggest a link between endosomal signalling of RTKs and cell migration[149].This finding was further developed by Assaker et al., who showed in Drosophila that fully functional trafficking between the plasma membrane and endosomes, involving both Rab5 and Rab11, was required to keep RTKs active at the leading edge of migrating border cells and consequently for directional cell migration[150].

As previously mentioned for EGFR, it is possible that a receptor can take various different internalisation routes, which can lead to different receptor fates[81]. TGF-β receptor has also been shown to internalise both through caveolin and clathrin dependent mechanisms[151]. Interestingly, clathrin-mediated internalisation into EEA1/SARA (Smad anchor for receptor

internalisation) -positive endosomes leads to continued signalling of TGF-β through Smad-2, while internalisation through cavelolin lipid rafts into caveolin-positive endosomes leads to receptor degradation[151].

Endosomal signalling has also been shown to occur from other non-tyrosine kinase receptors, including G-protein coupled receptors (GPCRs). For example, the inhibition of β2-adrenergic receptor endocytosis leads to a reduction in MAPK activation, while Shc and Raf activation are unaffected[141, 152]. Furthermore, Endo180, a collagen receptor, provides another example of a non-RTK that signals from endosomes. Endo180 mainly resides in endosomes due to its constant recruitment to clathrin-coated pits leading to its constitutive internalisation and recycling back to the cell surface[153]. In doing so, Endo180 brings collagen into endosomes, allowing collagen to be degraded and this accounts for remodelling of the extracellular matrix[154]. However, aside from this function of Endo180, it also has an important role in the activation of Rho, ROCK, Cdc42 and Rac during cell migration[155, 156]. Importantly, Endo180 needs to internalise and be present on endosomes for it to initiate its ROCK-dependent role in cell migration[155].

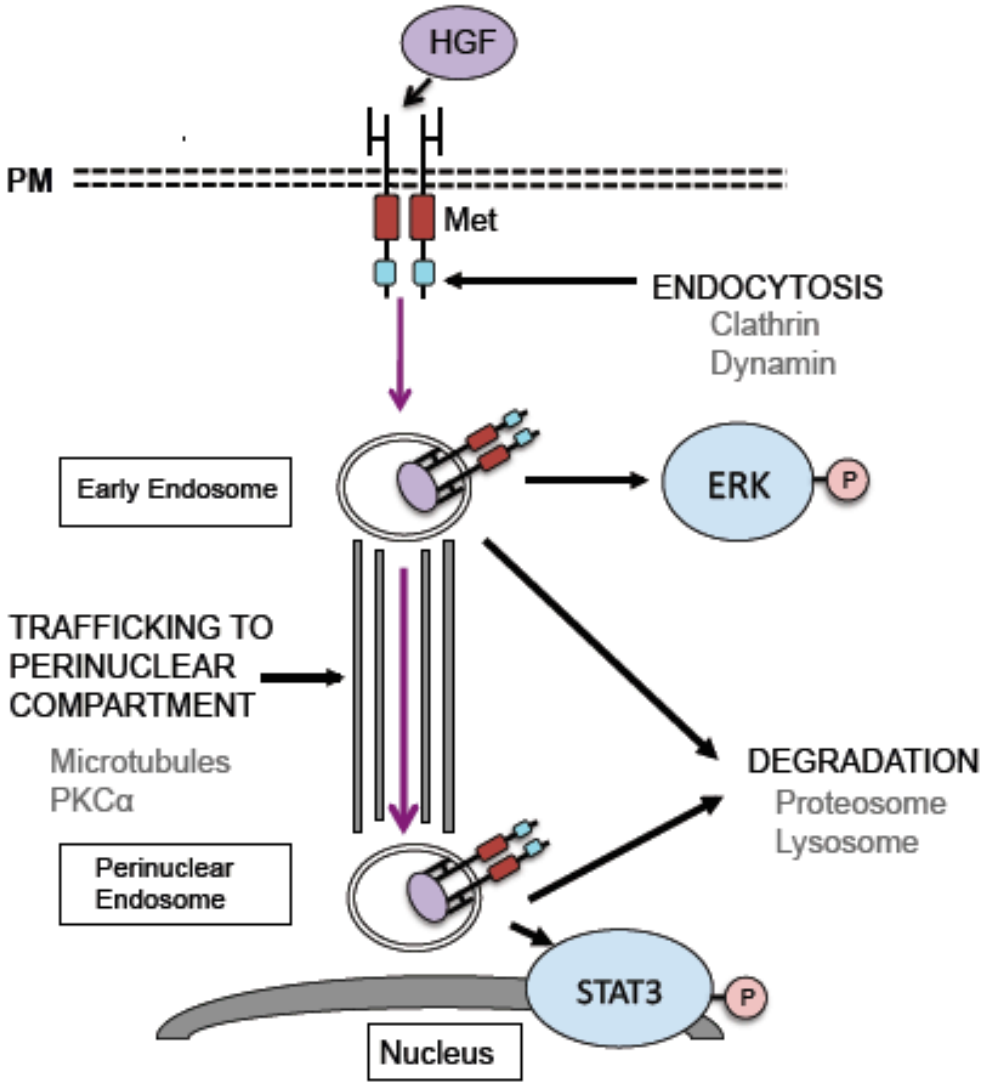


Figure 9: Trafficking and "endosomal signalling" of Met

Met is internalised by clathrin mediated endocytosis and traffics from early endosomes to a perinuclear compartment along microtubules. This traffic is promoted by PKCα activity. While in endosomes Met can continue to signal and is required for the full activation of ERK1/2 and STAT3[62, 157].

Based on Kermorgant S et al. Embo J, 2004[62]

3) Met endosomal signalling

Studies on the endosomal signalling of the Met receptor are just beginning to surface. Palamidessi et al. showed that clathrin-dependent endocytosis upon HGF (or Rab5 overexpression) is required for the activation of Rac[158]. Endosomes bring together Rac with its GEF, Tiam1, leading to its activation[158]. Once activated, Rac relocates to the plasma membrane where it carries out its role in actin remodelling and consequent cell migration[158]. However, in this study no link to Met localisation was made. Activation of Met leads to the downstream activation of a wide variety of signalling pathways and therefore some control over the spatial and temporal activation of the signalling molecules is necessary. Kermorgant et al. have demonstrated that Met undergoes "endosomal signalling"[62, 82, 157]. These studies show that endocytosis is required for full activation of the MAPK pathway and STAT3 upon HGF stimulation[62, 157]. Full activation of ERK 1/2 and resulting induction of wound healing upon Met activation depends on Met internalisation and trafficking to early endosomal compartments, but does not require Met trafficking to perinuclear endosomes[62]. Whereas, Met internalisation and trafficking to a perinuclear compartment is required for sustained STAT3 activation and nuclear accumulation, which is consequently required for HGF induced wound healing **(Figure 9)**[157].

Recently, our lab has shown that a constitutively active Met mutant has altered trafficking, which through increased endosomal signalling leads to increases tumourigenicity[159]. My contribution to this work is detailed in the Results section, but briefly this was the first study to make a direct link

between endosomal signalling of Met and tumourigenesis. As well as this initial “proof of principle” study I have also investigated this concept in a variety of other cancer cell models.

4) Mechanisms of endosomal signalling

Not much is known about how signalling complexes assemble on endosomes. One possibility is the recruitment of specific adaptors. Endosomes act as signalling platforms bringing together various components for signal generation. One such ‘scaffolding molecule’ is Appl1, a Rab5 effector molecule, found in endosomes, known as APPL endosomes[160], which later mature into EEA1 positive early endosomes[161]. Appl1 associates both with AKT and transmembrane receptors[162]. Its localisation on endosomes is required for full activation of the Akt pathway and cell survival during zebrafish development, making it the first study to investigate endosomal signalling *in vivo*[162]. MP1 (MEK1 partner) is another scaffold protein localised to late endosomes by the adaptor protein p14, where it binds to MEK1, activates ERK1/2 and controls EGF dependent proliferation[163]. As I mentioned earlier, endosomes have been shown to act as a scaffold for Rac to assemble with and be activated by its GEF Tiam1 upon HGF stimulation[158], however this study did not investigate Met localisation. Recent work from our lab has found that the presence of Met, together with PI3K and a specific Rac GEF, on perinuclear late endosomes is required for sustained Rac activation upon HGF in breast cancer cells (Menard et al. Paper in revision).

As well as acting as a scaffolding platform, endosomes also provide the acidic pH required for many signalling pathways[73], while also providing protection from phosphatases[157].

VI- Met in Cancer

In addition to the increased expression of Met in injured tissue, increased expression of Met / HGF occurs in many cancers and higher levels of Met expression often correlate with a poor prognosis[14]. This can occur through paracrine (HGF is released from the surrounding stroma) and autocrine (HGF is released from the tumour cells themselves) activation of the Met receptor[15]. Alternatively, if the receptor is overexpressed or there is an activating point mutation, Met can become activated without the need for ligand binding[14, 15]. The presence of HGF can initiate Met transcription[15] and, furthermore, some oncogenes, for example RAS, can bring about the transcription of Met and consequently lead to its overexpression[164].

Activating mutations of Met have been found in many cancers including ovarian, gastric, liver, kidney and head and neck cancer[14]. Thus, Met may provide a good target for treatment, to delay or prevent the progression of cancer.

1) Met in breast cancer

a) Breast cancer

Breast cancer is the leading cause of cancer-associated deaths in females in the Western world, despite huge advances in breast cancer detection and treatment[165]. Premalignant lesions begin with ductal hyperplasia which progress to carcinoma *in situ* and finally to invasive disease[165]. There are two main types of lesions; lobular lesions and ductal lesions, the latter of which is the most common[166]. These can progress to lobular carcinoma *in situ* (LCIS) or ductal carcinoma *in situ* (DCIS) respectively[166]. Here, cells have proliferated filling the ductal/lobular space but the myoepithelial and basement membrane layers remain intact[165]. Once this is broken, invasive carcinoma is present, either invasive lobular carcinoma (ILC) or invasive ductal carcinoma (IDC)[165, 166] and metastasis can occur **(Figure 10)**. There is no guarantee that carcinoma *in situ* will progress to invasive breast cancer and determining which patients with *in situ* disease will develop frank carcinoma is one of the problems currently facing breast cancer treatment[165, 166].

Gene expression studies have shown that there are 5 major types of breast carcinoma according to their molecular make-up; normal breast-like, HER2(ErbB2)-positive, basal-like and oestrogen receptor (ER) positive, which can be subdivided into 2 groups; luminal A (highest levels of ER expression) and luminal B (lower ER expression)[167]. The other three subtypes are ER negative[167]. The luminal A subtype has the best prognosis, while the HER2(ErbB2)-positive and basal subtypes have the least favourable outcome[168].

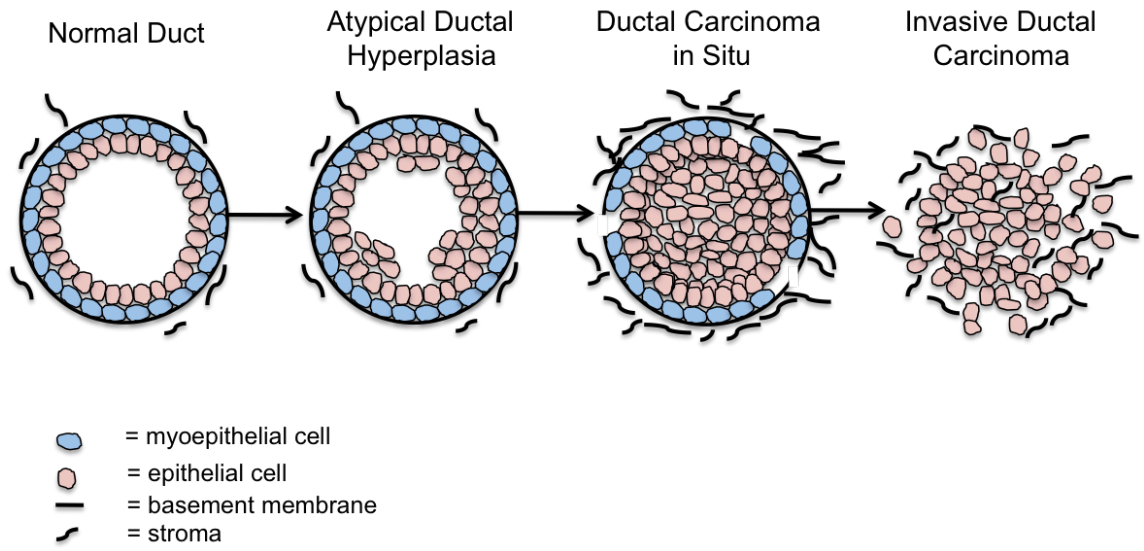


Figure 10: Breast cancer disease progression

A normal duct is surrounded by a layer of epithelial cells, enclosed by a layer of myoepithelial cells, which lie on the basement membrane. Atypical ductal hyperplasia is the result of proliferation of the epithelial cells, if this proliferation distends the intraductal space then ductal carcinoma in situ is present. This is often accompanied by a high level of stromal infiltrate and some of the myoepithelial/basement membrane layer may have started to break. Invasive ductal carcinoma is diagnosed if the epithelial cells have invaded through the myoepithelial/basement membrane layer.

Based on Polyak K et al, J. Clin. Invest, 2007[165]

Basal-like breast cancers are often “triple negative”: oestrogen receptor, HER-2 and progesterone-receptor negative[169]. Furthermore, individuals with BRCA1 mutations tend to develop basal-like breast carcinoma and this tends to occur at a younger than average age. There is therefore a desperate need for new treatment targets to be found for this type of breast cancer[12]. Basal-like breast cancer cells express cytokeratins 5, 14 or 17 as well as p53, c-kit, EGFR and p63[169]. CD44 has also been found to have increased expression in basal breast cancer cell lines and primary breast cancer tissue samples due to amplification of 11p13, which contains 19 genes including CD44[170]. However silencing of CD44 did not reduce cell survival or proliferation and so it is not believed to be the amplicon driver but rather associated with 11p13 amplification[170]. The collagen receptor Endo180 has been shown to be significantly correlated with basal breast cancer and significantly associated with shorter disease-free survival in a cohort of 880 invasive breast cancers[171].

b) Met in Breast Cancer

It is thought that Met is overexpressed in 20-30% of breast cancers[172-174]. A recent study has suggested that Met may be a marker for the basal breast cancer phenotype[175]. Met therefore may provide a valuable target for breast cancer therapy[176].

• *In Vitro* Studies

HGF has been shown to be produced by adipocytes in the breast stroma and to encourage the growth of a murine mammary carcinoma by the paracrine

activation of Met[177]. Met expression itself has been shown to be upregulated in various breast cancer cell lines[178] and its expression has been associated with angiogenic and lymphangiogenic factors in DCIS[179]. *In vitro* models of DCIS, using the two cell lines MCF10.DCIS and SUM102, have been used to demonstrate that HGF can promote the invasion of these cells by increasing their ability to degrade collagen type IV and the expression and secretion of uPA and uPAR [180].

Expression of Met may also be associated with drug resistance. Her2 positive breast cancer cells, which also express high levels of Met, have been reported to have reduced sensitivity to Trastuzumab (or Herceptin), an antibody against Her2, currently used in the clinic. Additionally HER2+ cells following Trastuzumab therapy appear to upregulate their Met expression. Combining Trastuzumab treatment with Met inhibition may benefit Her2 positive patients by sensitising the HER2+ cells to Trastuzumab[181].

• *In Vivo* Studies

A transgenic mouse model, where oncogenic Met was expressed in the mammary glands, led to the development of a range of mammary tumours with similarities to basal breast cancers[182]. Furthermore, studies have shown that 73% of mice with loss of BRCA1 and Trp53 had high expression of Met[183], which may correspond to the basal breast cancer phenotype.

In vivo nude mouse models of MDA-MB-231 xenografts injected with HGF or MRC-5 fibroblasts (which secrete high levels of HGF), were shown to have reduced growth including reduced angiogenesis following treatment with the

HGF antagonist NK4[184], thus demonstrating that inhibition of Met signalling in models of basal breast cancer may be an effective therapy.

• Clinical Studies

Several studies have been performed on clinical samples obtained from breast cancer patients and they indicate that Met may play a role in breast cancer progression. Overexpression of Met in 40 cases of primary breast cancer was found to be correlated significantly with the risk of progression of the disease and metastasis, reducing the disease free survival from 53 months (low Met expression) to 8 months[176]. In a study by Garcia et al. 270 out of 283 patients with invasive ductal carcinoma who died or had metastatic disease were positive for Met expression by immunostaining, while only 50 out of 633 patients who were alive or had no metastatic disease were positive[175]. Furthermore, the poor prognosis of individuals with overexpression of Met is independent of expression of Her2/neu[176]. Ghousssoub et al. studied 91 tissue samples from patients with invasive ductal breast carcinoma using immunofluorescence[174]. Strong expression of Met was found in 22% of these samples[174]. These patients had significantly reduced survival rates, from 89% 5-year survival in Met negative patients to 52% in Met positive patients[174]. This was found to be an independent prognostic factor that was as good as or better than, other prognostic factors such as lymph node status, age etc.[174].

• Summary

Therefore Met appears to be important for the formation and progression of breast cancer, as well as in the development of resistance to other TKIs (Tyrosine kinase inhibitors). Furthermore, it may be a particularly good target for the basal breast cancer sub-group. However, the studies mentioned above have only investigated Met expression and signalling. What about Met trafficking and endosomal signalling in such tumour cells?

c) Aberrant receptor trafficking in breast cancer

- Non-RTKs

A few studies have described altered receptor trafficking in breast cancer. The collagen receptor Endo180, has been shown to be upregulated in basal breast cancer as I mentioned previously. However, importantly, the internalisation of this constitutively recycling receptor is required for its role in increased tumour growth observed in mice[154]. The G protein associated receptor PAR-1 has been shown to have dysregulated trafficking by slow internalisation and recycling, that prevents it being sorted to lysosomes for degradation, leading to its persistent signalling[185].

- Modification of endocytic regulators

The first endocytic protein demonstrated to be involved in tumourigenesis was Hip1. Rao et al. showed that Hip1 expression was significantly higher in breast cancer tissue compared to normal breast tissue and this correlated with high EGFR expression. This correlated to increased EGFR expression in Hip1 overexpressing NIH3T3 cells *in vitro* as well as increased tumour

formation *in vivo*[186]. Since this study, other endocytic proteins have been found to be dysregulated in cancer. Analysis of microarray expression profiles of 737 primary invasive breast cancer cases identified two amplicons at 17q12 and 8p11-12 that occurred in tumours[187]. The 8p11-12 amplicon occurred in 17% of breast cancer cases analysed and the Rab-coupling protein (RCP) gene, involved in recycling, was found to be the gene most significantly associated with metastasis which was located in this region[187]. Overexpression of RCP led to the normal human mammary MCF-10A cells acquiring tumourigenic properties with increased activation of Ras and ERK1/2[187]. This is most likely due to changes in the trafficking of receptors, particularly recycling[187, 188]. The small GTPase Rab25, which also is involved in vesicle recycling of membrane receptors, including $\alpha 5\beta 1$-integrin in epithelial cells[189], has been shown to have increased expression in advanced breast and ovarian cancers[190]. The expression of Rab25 is associated with increased cell proliferation and survival *in vitro* as well as decreased overall patient survival[190]. Nevertheless, these studies on Hip1, RCP and Rab25 only *suggest* that receptor trafficking is modified. Hip1, RCP and Rab25 are all endocytic regulators that control the trafficking of many cell receptors including RTKs and so there could be numerous effects as a consequence of their modified expression.

- <u>RTKs</u>

In terms of aberrant trafficking of RTKs in breast cancer, very little has been published. The EGFRvIII mutant, found in many cancers including breast cancer[191, 192], lacks part of its extracellular domain and is unable to undergo

proper phosphorylation of its c-Cbl binding site (Y1045)[193]; thus, leading to a defect in ubiquitination, internalisation and degradation of the receptor[193]. Moreover, Rab11a, has been shown to be overexpressed in DCIS and to induce proliferation and increased MAPK signalling in MCF10A cells upon EGF stimulation, through recycling of EGFR[194].

- Summary

The studies on activated receptors, such as the EGFRvIII mutant and PAR-1 receptors, demonstrate persistent signalling due to a prolonged lifespan. They have decreased degradation through decreased internalisation or increased recycling. Therefore, so far nothing has been shown demonstrating a difference in RTK internalisation and trafficking towards endosomes or a direct role for *endosomal signalling* in breast cancer progression. Furthermore, there is no report of dysregulated trafficking or endosomal signalling of Met in breast cancer to our knowledge.

2) Met mutations in cancer

a) RTK mutations in cancer

Several RTK mutations have been discovered in cancer. They lead to RTKs being constitutively active and can confer drug resistance. For example, EGFR mutations in the kinase domain (exons 18-21) have been found in 10% of non-small cell lung cancers, mostly occuring in adenocarcinomas, which result in increased EGFR activation[195]. These mutations usually are sensitive to TKIs; however some mutations, such as the T790M mutation or insertion mutations in exon 20, lead to resistance to EGFR inhibitors[195].

The RTKs Kit and Ret contain a highly conserved kinase domain with Met and interestingly, mutations have been identified which correspond to the Met mutations I have been investigating. The constitutively activating M918T mutation located in the kinase domain of Ret is homologous to the M1268T Met mutation I will describe later, and has been found to be the cause of most cases of multiple endocrine neoplasia type 2B[196, 197]. Meanwhile, the constitutively activating D816Y/V in the kinase domain of Kit is homologous to the D1246N Met mutation, which I will also describe later, and has been found to occur in mastocytosis[198, 199].

The studies mentioned above are believed to indicate that Met mutations may be oncogenic due to their constitutive kinase activity. A few studies have described RTK mutants that have been found to have altered trafficking. The EGFR L858R and L816Q mutations in the activation loop of EGFR that were discovered in NSCLC lead to a defect in degradation due to an association with HSP90 and therefore are sensitive to treatment with HSP90 inhibitors[200]. The EGFRvIII mutant lacks part of its extracellular domain and is unable to undergo proper phosphorylation of its c-Cbl binding site (Y1045)[193]; thus, leading to a defect in ubiquitination, internalisation and degradation of the receptor[193].

Nevertheless, prior to the work carried out in our lab, no RTK mutations have been discovered that lead to altered trafficking corresponding to increased endocytosis and recycling, and which are responsible directly for their increased tumourigenicity.

b) Met Mutations in Cancer

• Identification of Met Mutations in Cancer

Mutations in Met have been identified in a variety of human cancers, including papillary renal-cell carcinoma, childhood hepatocellular cancer, gastric cancer and lymph node metastasis of head and neck squamous-cell carcinoma[16]. Recently, a somatic intronic mutation was discovered in lung cancer, leading to an alternative spliced Met transcript and consequent deletion of exon 14, encoding for the juxtamembrane domain[201].

Schmidt et al. discovered the first Met mutations in 1997 when they found germline and sporadic missense mutations in the tyrosine kinase domain in cases of papillary renal carcinoma[202]. In order to investigate whether these mutations could influence cancer progression, Jeffers et al. expressed them in NIH3T3 cells, which lack Met expression and which were selected for their low HGF production[203]. The mutations, which included M1268T, D1246H, Y1248H, Y1248C, V1238I, V1206L and M1149T, were found to constitutively activate Met, with M1268T the most strongly activated[203]. All of the mutants formed tumours in athymic nude mice more rapidly than those with wild type Met, with the M1268T mutants being the most tumourigenic both *in vitro* and *in vivo*[203].

• Mechanism of increased tumourigenicity of Met mutants

Recent work from our lab by Carine Joffre has advanced the understanding of the mechanisms of the increased tumourigenicity of the Met mutants. As mentioned briefly previously, it was found that while the mutants, M1269T

and D1246N, were more highly phosphorylated than the wild type Met stimulated with HGF, it was not only their constitutively high activation that was the cause of their high tumourigenicity.

The mutants were found to be constitutively internalised and recycled back to the membrane and to have a defect in degradation **(Figure 11)**. The result of this is the accumulation of the Met mutants on endosomes, where they continue to signal. There was found to be a requirement for endocytosis for Rac1 activation, suggesting that Rac1 is activated on endosomes by Met mutants. Blocking the endocytosis of the Met mutants was able to reduce cell migration and anchorage independent growth as well as tumour growth and *in vivo* metastasis of cells expressing these variants. Furthermore, blocking Met endocytosis had no effect on Met phosphorylation, thus the reduction in tumourigenesis observed by the alteration of mutant Met trafficking was independent of Met activation[159]. This demonstrates that the tumourigenicity of this Met mutant requires both constitutive Met activation *and* its accumulation on endosomes. Therefore inhibiting either of these characteristics reduces Met mutant tumourigenesis to an equal degree[159].

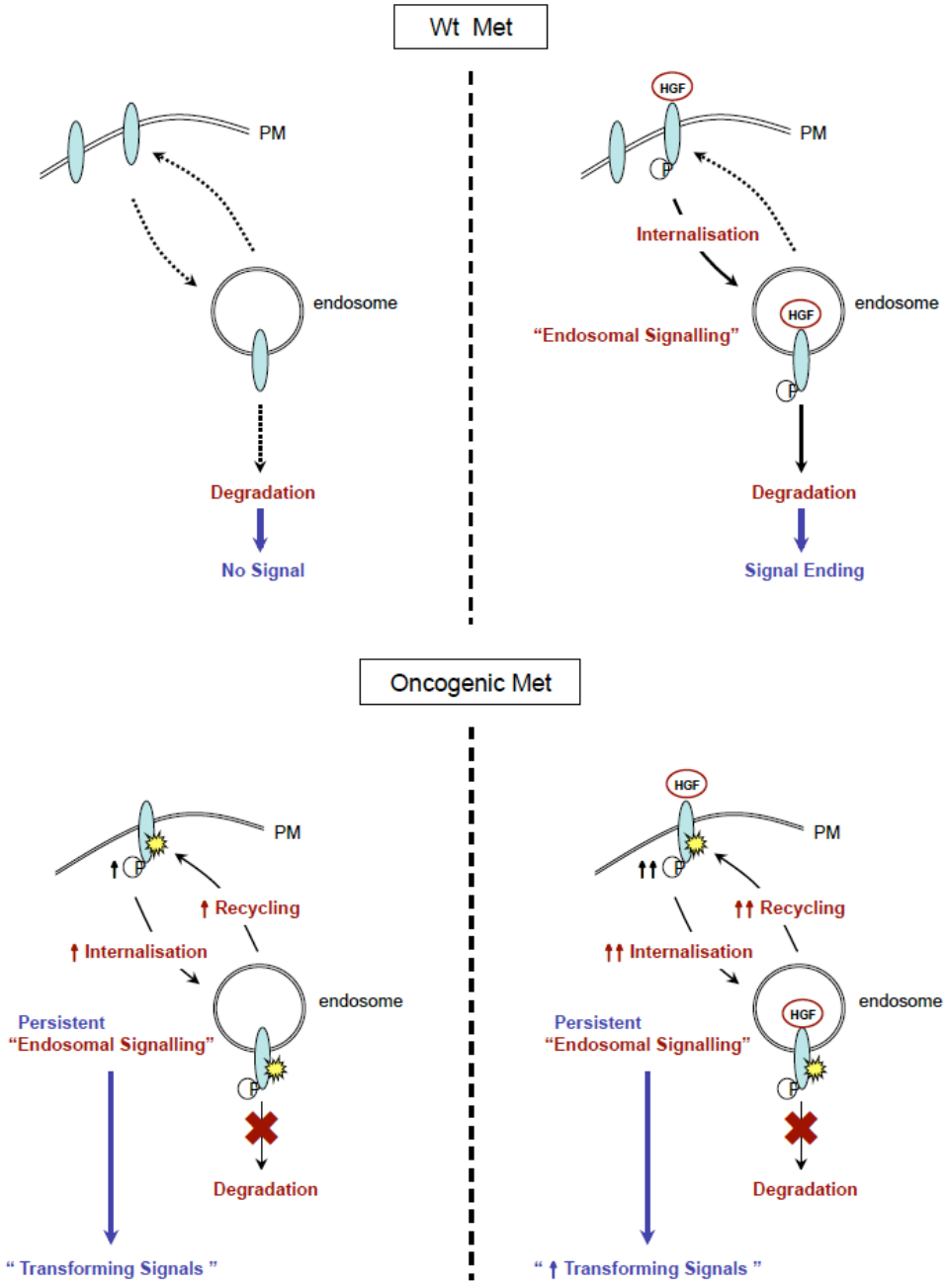


Figure 11: Model of Wt Met and oncogenic mutant Met signalling

Taken from Joffre C et al., Nat. Cell Biol., 2011[159]

VII- Targeting Met in cancer

1) Types of Met Signalling Inhibitors

Since Met signalling is involved in multiple human cancers, various inhibitors have been produced that target either Met or its ligand HGF. There are currently no Met inhibitors used as a first line treatment for cancer in the clinic. There are however many clinical trials, currently running, to evaluate their therapeutic potential.

a) 'Decoy' HGF

A truncated form of HGF, 'NK4' (N-terminal Kringle 4), was developed by Date et al.[204]. By binding to Met, it inhibits HGF-Met binding[204]. It has been shown to have anti-tumourigenic, anti-metastatic as well as anti-angiogenic effects in various human cancer models[205].

b) HGF antibodies

Amgen has produced quite a few monoclonal anti-HGF antibodies[206]. AMG102 is the best characterised and has proven to reduce tumour growth *in vitro* and *in vivo* working in synergy with Temozolomide or Docetaxel chemotherapeutic agents[207]. It is currently undergoing many Phase I and II clinical trials both as a monotherapy and in combination with chemotherapy in the treatment of various cancers including glioma, prostate cancer, colorectal cancer and renal cell carinoma[8].

c) 'Decoy' Met

Michieli et al. developed a soluble Met protein that matched the extracellular region of Met, thus lacking a kinase domain and the ability to signal[208]. Furthermore, the 'Met decoy' binds to both HGF and full size Met with high affinity, thus inhibiting signalling through both ligand binding and receptor dimerisation[208].

d) Met antibodies

The OA-5D5 monoclonal anti-Met antibody (also called METMab or Onartuzumab) was developed by Genentech and showed high efficacy *in vitro* and *in vivo* in U87MG glioma cells, which contain an HGF/Met autocrine loop, by inhibiting HGF-Met binding[8, 209]. It has shown very positive results in combination with the EGFR inhibitor Erlotinib in the treatment of non-small cell lung cancer (NSCLC) and has recently entered a Phase II trial in the treatment of triple negative breast cancer[8].

e) Non-ATP- Competitive Small Molecule Met Inhibitors

ARQ197, produced by ArQule, has been shown to inhibit Met over a panel of 230 kinases[210]. It inhibits the phosphorylation of Met and its downstream targets in a variety of cancer cell lines, where Met was either activated upon HGF stimulation or independently of HGF[210, 211], although it more favourably inhibits the inactive form of Met[212]. It does so by preventing ATP binding, but through a novel non-competitive method[8] It binds the ATP-binding cleft and an unique hydrophobic pocket[8, 212]. ARQ197 binding makes the ATP binding site non-polar and unsuitable for ATP binding, but it also stabilises Met in its

inactive conformation[212]. ARQ197 has produced promising results in phase II clinical trials treating NSCLC patients together with erlotinib and, as a result, ARQ197 combined with erlotinib has recently moved into phase III clinical trials[8].

f) ATP- Competitive Small Molecule Inhibitors

• Met inhibitors

This class of inhibitors act by competing with ATP for the activation of Met. K252a was the first to be described in the 1990's and it is an analogue of Staurosporine[45, 213]. Despite not being aimed specifically at Met it was found to efficiently inhibit Met activation of wild type and, particularly, mutant (M1268T) Met activity[45, 214]. More recently, more specific Met inhibitors, unrelated to K252a, have been developed. The first to be developed, SU-11274 and PHA-665752, are structurally related, both containing an indolin-2-core[45] **(Figure 12)**.

SU11274 was developed in 2003 by Sugen (now Pfizer)[215]. It was found to be 50-fold more active to Met than a panel of other kinases with an IC_{50} against Met activity of 20nM[216]. *In vitro* assays demonstrated that SU11274 inhibits Met phosphorylation as well as downstream signalling and cell viability[217].

PHA-665752 was developed around the same time as SU11274 also by Sugen (now Pfizer)[218]. PHA-665752 inhibited Met activity with an IC_{50} of 9nM and was also found to be 50-fold more sensitive against Met than a panel of

other tyrosine kinases[218]. *In vitro* assays demonstrated that PHA-665752 inhibits Met phosphorylation as well as downstream signalling[218, 219] and in doing so inhibited cell proliferation, survival, migration and invasion[218]. Furthermore, *in vivo* assays with PHA-665752 led to a reduction in either tumour volume or growth in the Met dependent cell lines GTL-16 (express high levels of constitutively active Met) and S114 (NIH3T3 cells that have been engineered to express high levels of HGF together with Met)[218]. It also led to a reduction in the growth of small cell lung[56] cancer (NCI-H69) and non-small cell lung cancer (NCI-H441) xenografts, which express high levels of Met[220, 221], through inhibition of both tumour cell growth and angiogenesis[56]. Finally, tumours containing Met amplification have been shown to be especially sensitive to PHA-66572[222] as well as those containing the oncogenic TPR-MET form of Met[219]. Development of resistance to PHA-665752 has been described *in vitro* and *in vivo* in Met dependent GTL-16 cells by activation of HER family members or amplification of KRAS and Met itself[223, 224].

However, the major problem with both SU-11274 and PHA-665752 is that both drugs have poor bioavailability, making their use *in vivo* limited[225].

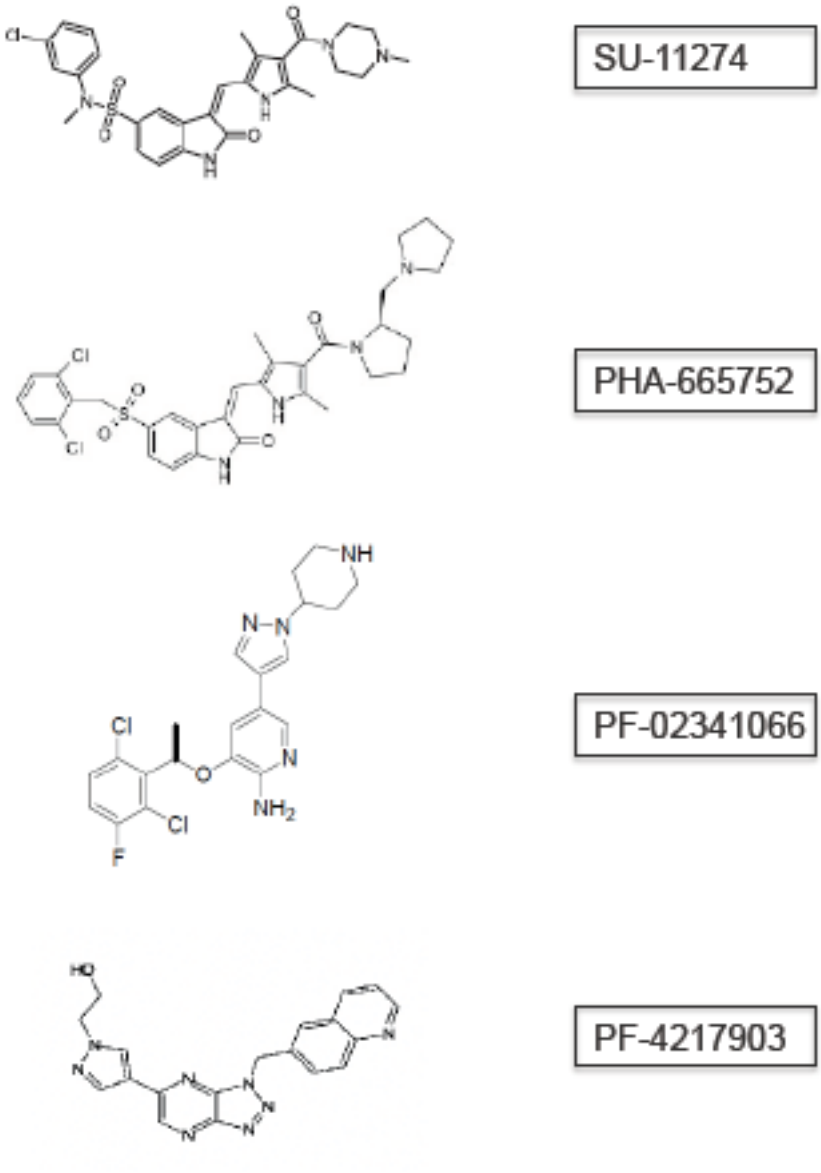


Figure 12: Small Molecule Met Inhibitors

The structure of SU-11274, PHA-665752, PF-02341066 and PF-421709 developed by Pfizer. The structurally related SU11274 and PHA-665752 have a common indolin-2-core. The more recently developed PF-02341066 and PF-4217903 are based on the 3-benzyloxy-2-aminopyridine chemical series and triazolopyrazine chemical series respectively.

Adapted from Wang X et al, Mol. Cancer Ther., 2003[215], Christensen et al, Cancer Res., 2003[218], Zou HY et al, Cancer Res., 2007[225], and Timofeevski et al., Biochem, 2009[226].

PF-2341066 is an orally available compound developed in 2007 by Pfizer. The development of this novel Met inhibitor was based on the crystal structure of Met with PHA-665752 bound in the kinase domain[227] and belongs to the 3-benzyloxy-2-aminopyridine chemical series[226]. It is a much more potent and specific inhibitor of Met activity than SU11274 and PHA-665752, with 100 fold selectivity for Met over 90% of the 120 kinases tested[225]. However, PF-2341066 inhibits the activation of both Met and ALK (Anaplastic Lymphoma Kinase) and their downstream signalling pathways in a dose dependent manner. *In vivo* studies showed that treatment with PF-2341066 inhibited tumour growth of both Met (GTL-16) and HGF (U87MG) dependent tumours as well as causing regression of some tumours, including non-small cell lung cancer (NCI-H441)[225]. The mechanisms for this inhibition were found to be through a decrease in proliferation and an increase in apoptosis of the tumour cells as well as a decrease in angiogenesis[225].

PF-4217903 is another more recent orally available compound from Pfizer, belonging to the triazolopyrazine chemical series. It potently inhibits Met activation and was found to be greater than 1000 fold selective compared to a panel of 208 other kinases[226]. Its potency against wild type Met is similar to PF-2341066 and it has demonstrated increased activity against several Met mutants[226].

Although here I have outlined those small molecule Met inhibitors relevant to my study, there are a variety of additional inhibitors that have been produced by multiple pharmaceutical companies recently. I have summarised the

variety of Met inhibitors that have entered clinical trials in Table 1.

Some are broader inhibitors targeting Met and other RTKs such as VEGFR. This approach of targeting multiple kinases can be very effective. An example of this is XL184 (cabozantinib), which targets many RTKs including MET, VEGFR2, and RET. In a phase I trial of patients with metastatic or unresectable medullary thyroid cancer, 49% had tumours that underwent shrinkage and 41% had stable disease for a minimum of 6 months. These positive results have led to XL184 entering Phase III trials.

Type	Name	Company	Target	Trial	Reference
HGF antibodies	AMG102	Amgen	HGF	Phase I/II: Solid tumours, Renal cell carinoma, Prostate cancer, Gastric cancer, Colorectal cancer, Malignant glioma, Small cell lung cancer and Ovarian cancer	207
	SCH900105/ AV-299	Shering/Aveo	HGF	Phase I/II: Various solid tumours, Non-small cell lung cancer, Multiple myeloma and Lymphoma.	211
Met antibodies	OA-5D5	Genetech	Met	Phase I: Various solid tumours and Non-small cell lung cancer	209
	Tak-701	Millenium	Met	Phase 1: Various solid tumours	211
Non-ATP-competitive small molecule inhibitor	ARQ197	ArQule	Met	Phase I/II: Solid tumours, Renal cell carinoma, Pancreatic adenocarinoma, Hepatocellular carcinoma, Non-small cell lung cancer and Clear cell sarcoma	211
ATP-competitive small molecule inhibitor	PF234-1066	Pfizer	Met/ ALK	Phase I/II: Various solid tumours, Non-small cell lung cancer, CNS tumours and Anaplasticlarge-cell lymphoma	225
	PF-4217903	Pfizer	Met/ ALK	Phase I: Various solid tumours - suspended	226
	SGX523	SGX Pharmaceuticals	Met	Phase I: Various solid tumours - terminated	211
	MK2461	Merck	Met, KDR, FGFR1-3, and Flt 1,3,4.	Phase I/II: Various solid tumours	211
	MP470	SuperGen	Met, Ret, Rad51, mutant forms of Kit, PDGFR, Flt-3	Phase I: Various solid tumours- one study terminated	211
	XL880/ GSK1363089	Exelixis/Glaxo Smith Klein Beecham	Met, Ron, VEGFR1-3, PDGFR, Kit, Flt-3, Tie-2	Phase I/II: Solid tumours, Renal cell carinoma, Gastric cancer, Squamous cell carcinoma of head and neck.	211
	AMG208	Amgen	Met	Phase I: Various solid tumours	211
	JNJ-38877605	Johnson	Met	Phase I: Various solid tumours	211
	EMD1214063	Merck Serono	Met	Phase I: Various solid tumours	211
	XL-184	Merck Serono	Met, VEGR2, Ret	Phase I/II/III: Various solid tumours	8

Table 1: Met inhibitors in clinical trials

VIII- Integrins and RTKs

Integrins are a family of transmembrane receptors that form heterodimers from an α and a β subunit. In mammalian cells there are 18 different α subunits that can bind non-covalently to one of 8 β subunits, the combinations of the two subunits form 24 different integrins **(Figure 13)**[228].

The β1 subunit is ubiquitously expressed and is the β-subunit of most ECM binding integrins as it forms the largest subgroup as it can associate with 12 α partners[229]. It has roles in foetal development, cell migration, differentiation, haematopoiesis, assembly of the ECM and tumourigenesis[230].

Integrins directly link the cell cytoskeleton to various components of the extracellular matrix (ECM), consisting of collagens, laminins and proteins containing the RGD (Arginine-Glycine-Aspartic acid) motif (including fibronectin and vitronectin)[228, 231]. They therefore play a major role in cell adhesion and also in cell migration by supplying the necessary traction but as well through remodelling of the ECM via relocalisation and activation of various proteases, including MMP2, MMP9 and uPAR, at the migrating edge of the cell[232, 233].

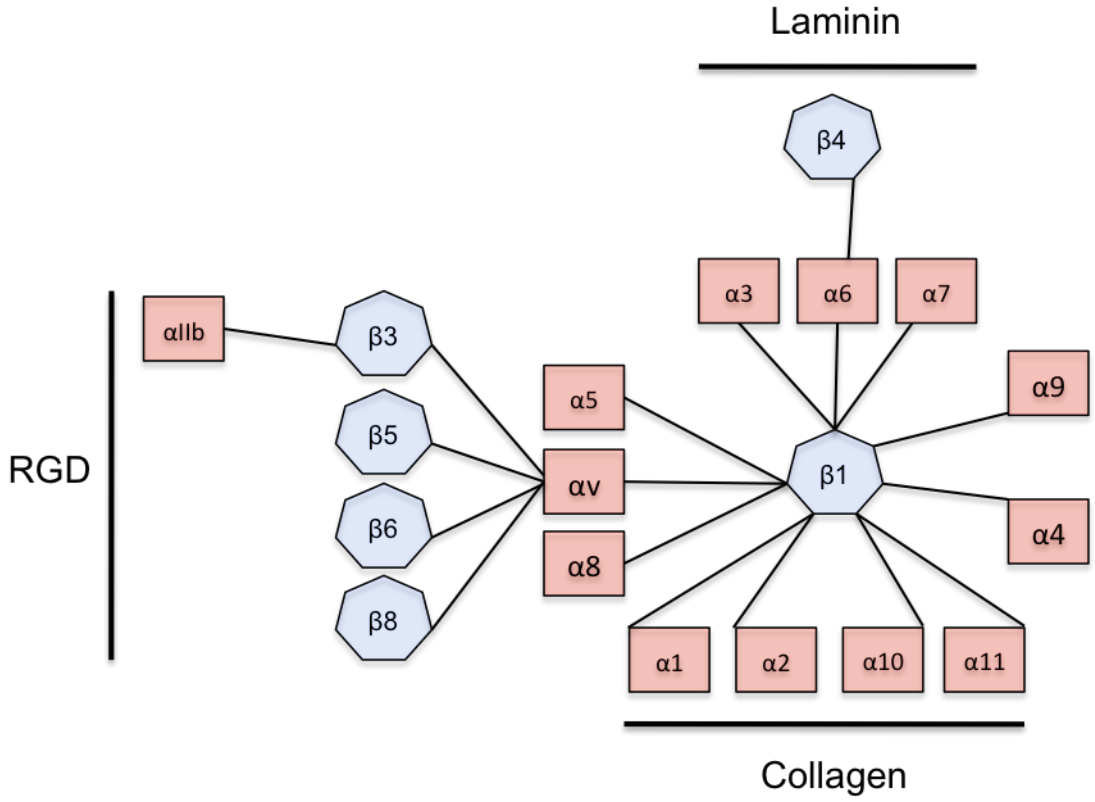


Figure 13: Integrin heterodimers found in adherent cells

This diagram outlines all the integrin heterodimers that can occur in adherent cells and to which ligands in the extracellular matrix they bind.

Based on Hynes R.O., Cell, 2002[228]

1) Integrin activation

Integrins have several possible conformations, often discussed in terms of integrin activation, which affect their ligand-binding affinity[234]. There are high-affinity, intermediate-affinity and low-affinity conformations and within a cell a dynamic equilibrium between the conformational states of the integrin population is present[231]. Once this equilibrium shifts towards an increase in the high-affinity conformation then this is characterised as integrin activation[231]. The proportion of integrins in active and inactive conformations varies depending on cell type and cell behaviour. For example, a higher proportion of integrins are in the inactive conformation in circulating platelets but integrin activation occurs upon platelet activation, while a higher proportion of integrins are in the active conformation in adherent epithelial cells but will cycle between inactive and active states during cell migration[231].

The low-affinity (inactive) conformation of integrins has a bent shape **(Figure 14)**, the intermediate-affinity conformation has an extended shape while the high affinity (active) conformation has an extended shape and is bound to its ligand, although it is possible for the low-affinity form to also be ligand bound[235]. This change in conformation is thought to occur with a separation of the cytoplasmic tails of the alpha and beta subunits.

Integrin activation can occur either through extracellular events, including ligand binding, or through changes in the cytoskeleton as discussed below[231].

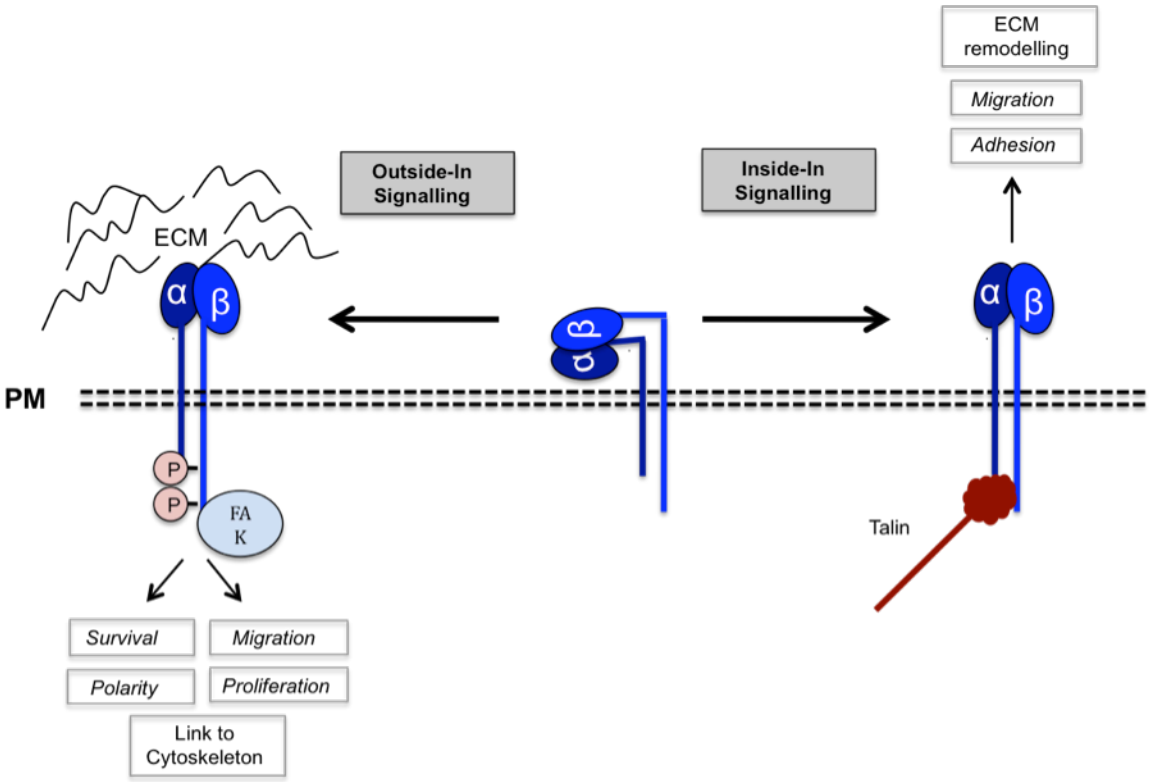


Figure 14: Integrin activation and signalling

The low-affinity (inactive) conformation of integrins has a bent shape, while the high affinity (active) conformation has an extended shape and is bound to its ligand. “Outside-in signalling” occurs when ligand binding of an integrin heterodimer leads to tyrosine phosphorylation of two motifs in the cytoplasmic tail, which can lead to the phosphorylation of intracellular molecules such as FAK . “Inside-out signalling” occurs through the binding of the intracellular cytoskeletal protein Talin, which increase ligand affinity through altering the integrin conformation.

Based on Shattill 2010[235]

2) Integrin Signalling

In fact, integrins are known to play a role in cell signalling. Although they themselves lack kinase activity, their binding to molecules in the ECM leads to the recruitment and activation of various intracellular signalling molecules at sites called focal adhesions[232].

a) "Outside-In Signalling"

There are two regions of the β-integrin subunit cytoplasmic tail that are involved in integrin signalling; the membrane-proximal NPxY motif and the membrane distal NxxY motif[231]. Tyrosine phosphorylation of these regions occurs after ligand binding or through interaction with RTKs as described in Part 4 and leads to altered binding affinity of interactors such as Talin and Tensin[231]. The majority of integrins are able to activate Focal Adhesion Kinase (FAK) at Y397 through the cytoplasmic domain of the β subunit[236]. This leads to formation of a FAK-Src complex, which consequently phosphorylates the scaffolding molecules p130Cas and paxillin[232, 233, 236]. This can lead to the downstream activation of AKT/PKB, MAPK/ERK as well as Rac[233], all of which are known to contribute to cell migration. Moreover, recruitment of talin, paxillin, vinculin, tensin and α-actinin to focal adhesions provides a link to the actin cytoskeleton[232].

b) "Inside-Out Signalling"

Furthermore, integrins can signal in a bidirectional manner as changes in the cytoskeleton, are able to alter the ligand-binding affinity of integrins, termed "inside-out signalling"[228, 231]. This occurs through the binding of the

intracellular protein Talin, a cytoskeletal protein that is linked to the actin cytoskeleton, to the cytoplasmic domain of the β integrin subunit[235, 237, 238]. In doing so, it is thought that Talin disrupts an electrostatic interaction between the α and β cytoplamic tails, altering the conformation of the integrin and increasing its ligand affinity[235, 238]. Another group of β integrin subunit binding proteins that have been found to be involved are the Kindlins, which bind to a more distal region of the β integrin cytoplasmic tail compared to Talin, and can help regulate integrin activation together with Talin[235, 238].

3) Integrin trafficking

Fibonectin-binding integrins were the first integrins shown to undergo endocytosis and recycling in CHO fibroblasts in a study published in 1989 by Bretscher et al.[239]. It has recently become clear that integrin trafficking is an important process enabling the formation and dismantling of focal adhesions, ECM remodelling and the redistribution of integrins in the plasma membrane[231, 240]. These functions consequently allow cellular adherence and migration. Integrin internalisation can occur via clathrin-dependent and –independent mechanisms[231, 240]. Some integrins use a specific pathway, while others can use several that can sometimes depend on the activation state of the integrin[231, 240].

Arjonen et al. recently showed in cancer cells that the active and inactive forms of β1-integrin follow different trafficking pathways[241]. Both the active and inactive forms of β1-integrin internalise via clathrin and dynamin dependent mechanisms. However the active form has a faster overall rate of

internalisation and traffics to a Rab7 positive compartment with its ligand, while the inactive form undergoes a slower internalisation and is quickly recycled back to the Arf6 positive protrusions of the plasma membrane in a Rab4 and F-actin dependent manner[241]. The consequence is that a greater proportion of β1-integrin in the active conformation is present in the cytoplasm with the majority of inactive β1-integrin at the plasma membrane[241]

In recent years it has emerged that integrins can undergo altered trafficking in cancer cells. As I have previously mentioned, the GTPase Rab25 has been associated with tumour progression. In fact, Rab25 can directly associate with β1-integrin and supports α5β1 integrin dependent invasion through fibronectin by maintaining a population of recycling α5β1 integrin at pseudopodial tips[189].

4) Integrin-RTK cross-talk

There are various ways in which RTKs and integrins have been shown to crosstalk. They can associate together at the plasma membrane upon integrin binding to the ECM or they can crosstalk via converging downstream signalling molecules[242].

a) Cross-talk in RTK-integrin Signalling

- RTK influence on integrin signalling

The "classical" model of cross-talk between RTKs and integrins occurs when growth factors stimulate integrin activation[243]. Stimulation of cells with growth factors such as EGF can lead to the disassembly of focal adhesions[244],

which are locations of integrin based ECM attachment to the actin cytoskeleton involving many molecules[232]. Furthermore activation of RTKs by their growth factors can also alter integrin expression levels[243].

- Integrin influence on RTK Signalling

The fact that integrins can influence RTK signalling has been much less studied and is a relatively new concept. Most of the studies in this area have focused on the adhesion dependent activation of RTKs. It has been shown that integrins are capable of activating RTKs through binding to the extracellular matrix **(Figure 15)**, independently of their ligand. For example ligand binding of β1 or αv integrins leads to a transient EGFR phosphorylation in ECV304 endothelial cells[245]. Furthermore, adhesion leads to an increase in the number of EGFR molecules on the cell surface and, importantly, EGFR and β1 have been found to form a complex at the plasma membrane[245, 246]. The cytoplasmic domain of β1 as well as c-Src and p130Cas are required for this integrin mediated EGFR phosphorylation, while p125 FAK has been found not to be required[246].

The studies just mentioned are all dependent on cell adhesion. Interestingly, a study by Guo et al. showed that β4-integrin is required for ErbB2 dependent mammary tumourigenesis *in vivo,* independent of α6β4-integrin ligand binding [247]. ErbB2 was found to associate with the signalling domain of β4-integrin and induce β4-integrin phosphorylation through Src-family kinases [247]. Furthermore, β4-integrin was found to be required for Src-family

kinases induction of ErbB2 phosphoryaltion, so β4-integrin and ErbB2 require each other for full signalling capability[247]. The β4-integrin signalling domain was found to increase c-Jun activation, through JNK nuclear translocation as well as Stat3 activation; events which promote hyperproliferation and cell junction disruption respectively[247].

b) Cross-talk in RTK-Integrin Trafficking

The fact that RTKs and integrins can regulate each other's trafficking is a relatively new concept, but it is particularly relevant given the importance of the trafficking of both molecules in cancer cells.

- RTK influence on integrin trafficking

A few studies have demonstrated that RTK activation can regulate integrin trafficking[248]. For example, stimulation of HeLa cells with foetal calf serum or EGF leads to the recycling of internalised β1-integrin in a Rab11 and Arf6 dependent manner[248, 249]. Moreover, activation of VEGFR1 has been demonstrated to transport αvβ3 to the plasma membrane from early endosomes in a Rab4 dependent manner[250]. This was shown to be important for ECM remodelling and *in vitro* capillary branching[250]. In a similar manner, stimulation of NIH3T3 cells with PDGF is required for the recycling of αvβ3 to the leading edge of cells and is consequently important for cell migration[251].

- Integrin influence on RTK trafficking

Alternatively, integrins also have a role to play in RTK trafficking. A study by Caswell et al showed that inhibition of αvβ3 in cancer cells brings about

increased recycling of α5β1 and EGFR, which is dependent on RCP, leading to increased EGFR signalling and cell migration[252]. Interestingly, inhibition of αvβ3-integrin using Cilengitide, has also been shown to promote Rab4-driven recycling of VEGFR and β3-integrin, protecting VEGFR from degradation[240, 253]. These results might help to explain why Cilengitide has not had the hoped for effect in blocking angiogenesis in cancer patients in clinical trials.[253] The concentration required to inhibit angiogenesis and tumour growth is high, once the plasma concentration falls to 0.2-20nM it becomes tumour-promoting[253].

- Co-trafficking between RTKs and integrins

Indirect cross-talk between RTKs and integrins has also been described through co-trafficking along a similar pathway in cells containing mutant p53. The tumour suppressor protein p53 acts by inducing cell death following cell stress or damage and mutations in the p53 gene lead to a loss of protein function in approximately 50% of cancers[254]. However recent evidence has emerged that mutant p53 can have added pro-tumourigenic roles in addition to the loss of tumour suppressor functions. Muller et al. demonstrated that the presence of mutant p53 leads to an increased recycling of both α5β1 and EGFR, in an RCP dependent manner[255].

5) Integrin-Met cross-talk

Numerous studies have reported integrin-Met cross-talk, however few describe mechanisms for how the cross-talk occurs, with none investigating where the cross-talk occurs.

a) Cross-talk in Met-integrin signalling

- The influence of Met on integrin signalling

HGF can increase α2-integrin expression in adherent MDCK cells[256]. HGF has also been shown to activate αvβ3 integrin in normal thyroid cells (HTU-5) promoting cell attachment and spreading[257]. These studies demonstrate that Met activation can play a role in integrin signalling in an anchorage dependent manner.

Importantly, Met has been shown to associate specifically with α6β4 (not with EGFR or Ron) independently of both Met and integrin ligand binding[258]. In fact, this study by Trusolino et al. published in 2001 was the first to suggest that integrins could have a role in cell functions that was independent of ligand binding. They demonstrated that the activation of Met leads to the phosphorylation of the cytoplasmic tail of the β4 integrin subunit[258] which is required to sustain Met dependent anchorage independent growth, protection from apoptosis and *in vivo* metastasis[258]. These biological functions require the phosphorylated β4 to bind to Shc, which can then activate the MAPK pathway and may also activate PI3K[258]. Therefore in this situation the β4-integrin is acting as a signalling scaffold molecule to enhance Met signalling rather than as an adhesion receptor. Furthermore, phosphorylation of β4 integrin on Tyr1257, 1440 and 1494 following Met activation leads to the binding of β4 integrin to Shp2, with Tyr 1440 being necessary[259]. This leads to activation of Src, which phosphorylates Gab1 on the residues involved in Grb2 binding[259]. Both Shp2 and Src are required for anchorage independent growth.

- The influence of integrins on Met signalling

Cellular adherence was originally shown to induce Met phosphorylation in tumour cells by Wang et al.[260]. More recently Mitra et al have reported that binding of α5β1-integrin to fibronectin initiates a direct association between α5-integrin and Met, leading to HGF independent Met phosphorylation and a consequent association between Met and Src in ovarian cancer cells[261]. However, unlike the study by Trusolino et al., these studies were shown to be all dependent on cell adhesion.

b) Cross-talk in Met-Integrin Trafficking

- Co-trafficking between Met and integrins

In a similar way to EGFR, mutant p53 also increases Met signalling as well as Met dependent cell migration and invasion. This is due to increased recycling of both Met and α5β1, both of which were found to interact with RCP[262]. However, this study did not show whether Met and α5β1 recycle and co-localise together or separately.

c) Summary

So far no studies have yet shown that Met can directly alter integrin trafficking or *vice versa*. It is not known whether the ubiquitously expressed β1-integrin could promote Met signalling as α6β4 integrin does. Furthermore, the location of Met-integrin cross-talk and whether cross-talk could occur on endosomes has not been determined.

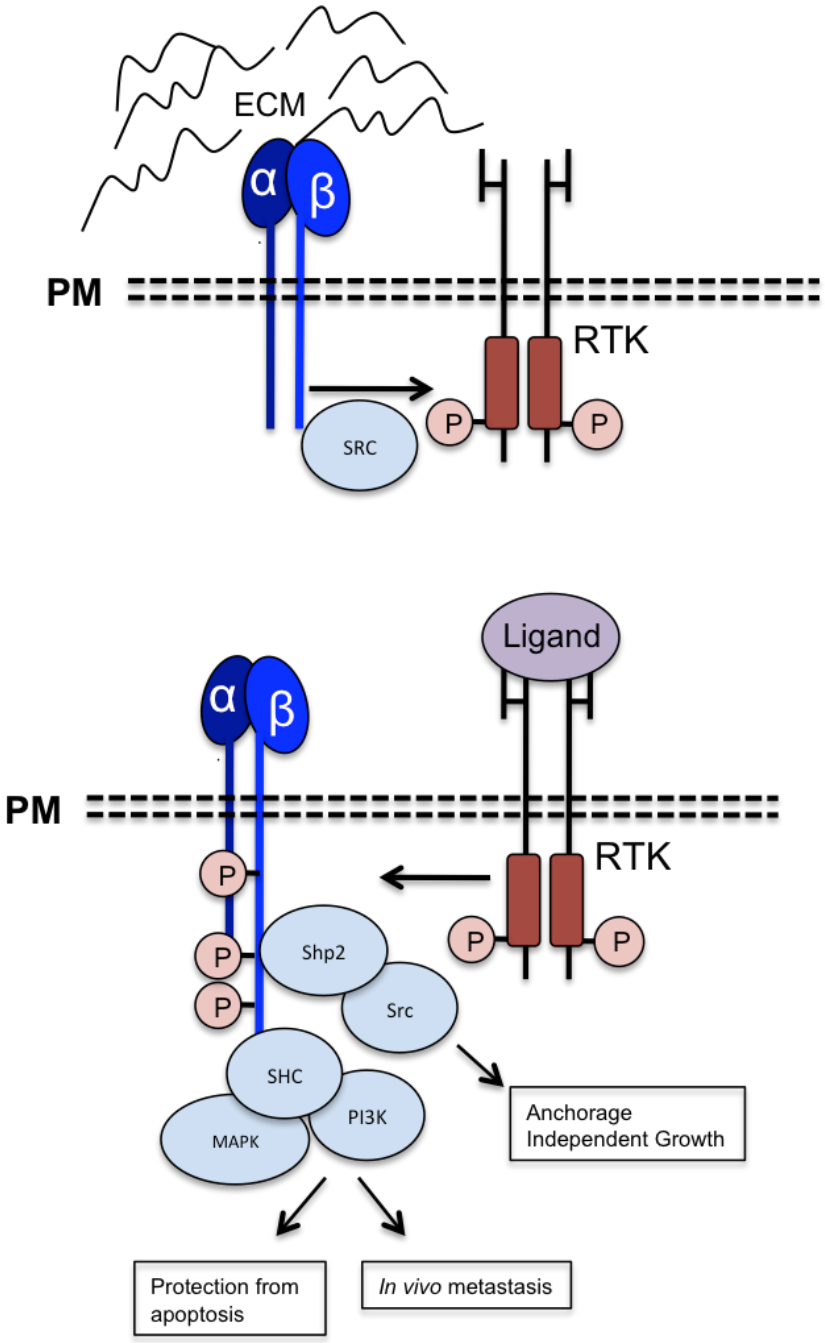


Figure 15: Integrin – RTK crosstalk.

Ligand binding of integrins can induce phosphorylation of RTKs. Additionally ligand binding of RTKs can lead to phosphorylation of the β1-integrin cytoplasmic tail, which can then act as a signalling scaffold for RTK signalling.

Based on Guo W et al, Nat Rev Mol Cell Biol, 2004[233]

6) Targeting integrins in cancer

Many integrins have been found to be overexpressed in a variety of human tumours; the expression of α4β1, α5β1, α6β4, αvβ3, αvβ5 and αvβ6 have all been associated with tumour progression, while α2β1 may behave in an opposite way as a tumour suppressor[232]. As a consequence of this, the pharmaceutical industry is trying to target these cell surface receptors. Cilengitide, developed by Merck KGaA, is an αv inhibitor which is showing positive results against glioblastoma in clinical trials and is consequently undergoing a Phase III trial[232]. Alternatively, integrin expression in tumours could potentially be used for tumour imaging or to enable specific drug delivery[232].

AIMS OF THE PROJECT

The objectives of my thesis were to investigate the potential role of Met signalling, trafficking and endosomal signalling in cell transformation, migration and in the development and progression of cancer to metastatic competence using several different models.

Through overexpression, Met is thought to play a role in breast cancer progression[175, 176], however precise mechanisms are unknown. In particular, Met trafficking and potential endosomal signalling in breast cells has not been documented.

My first aim, therefore was, to investigate mechanisms of Met signalling in breast cancer progression using basal breast cancer cell lines, ranging from a normal to a pre-invasive and finally to a highly aggressive phenotype, expressing endogenous WT Met (MCF10A, MCF-10ADCIS.com, MDA-MB-468 and MDA-MB-231). I compared Met signalling and subsequent migration of these cells, Met trafficking and the potential role of Met endosomal signalling in the stimulation of their migration. I extended this study to investigate Met expression and localisation in a series of normal, ductal carcinoma in situ (DCIS) and invasive ductal carcinoma breast tissue samples.

Met also can play a role in cancer progression through activating mutations in the kinase domain that have been identified in various human cancers[202]. Through transferring these mutations into cell models, it was shown that they

lead to Met constitutive activation and cell transformation *in vitro* and *in vivo*[203]. The high activation status of these Met mutants has been assumed to be the cause of their oncogenicity. However, the trafficking / endosomal signalling and the potential link with the oncogenicity of such mutants had, until now, not been investigated.

Working alongside Dr. Carine Joffre, who has found that these mutants present an increased internalisation and recycling and a defect in degradation, my second aim was therefore to investigate the signalling, particularly the endosomal signalling, of two oncogenic Met mutants, M1268T and D1246N, previously identified in renal cell papillary carcinoma. We used, as a model, NIH3T3 cells expressing Met Wt or mutants. My personal contribution included firstly testing the sensitivity of various small molecule Met inhibitors on these Met mutants. Secondly, I investigated the mechanisms of Met endocytosis of the Met mutants' and I attempted to restore the mutants degradation using HSP90 inhibition. My aim was then to observe the consequences of both inhibiting Met endocytosis and restoring Met degradation on Met dependent cell transformation both *in vitro* and *in vivo*.

It is known that Met undergoes cross-talk with integrins in both anchorage dependent and independent conditions[258, 260, 261]. However, whether β1 integrin specifically could promote Met signalling has still to be discovered. Furthermore, the localisation of Met-integrin cross-talk or whether one molecule can directly influence the trafficking of the other is not yet fully known.

My final aim was, therefore, to investigate initially the role of β1-integrin in

Met signalling and cell function in two cell models and furthermore to examine the location of this cross-talk. The first is a cell model consisting of the β1-integrin null GD25 cells, the GD25- β1A (containing β1-integrin) cells and GD25-YYFF (containing β1-integrin with mutations in two crucial tyrosines in the NXXY motif of the β1 cytoplasmic tail) cells. The second cell model consists of HEK-293 cells containing a tetracycline inducible constitutively activated Met-GFP, which induces a loss of cellular adherence and growth of the cells in suspension. This therefore provides us with the opportunity to observe the role of β1-integrin in Met signalling in unanchored cellls. Finally I investigated whether β1-integrin was required for Met dependent tumourigenesis *in vivo* using the previously described Wt and M1268T Met mutant expressing NIH3T3 cells.

MATERIALS AND METHODS

1) Cell Lines and Cell culture

All cell lines were certified by STR (Short Tandem Repeats) DNA profiling, which was performed by the Barts and The London Genome Centre in May 2011.

The MDA-MB-231 and MDA-MB-468 cell lines (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, PAA) containing 1% L-glutamine and 10% foetal bovine serum (FBS). The MCF-10ADCIS.com cell line (obtained from Dr. Steve Santner) was cultured in 1:1 DMEM: Ham's F12 (PAA) containing 1% L-glutamine and 5% horse serum (HS). The MCF10A cell line (ATCC) was cultured in 1:1 DMEM: Ham's F12 (PAA) containing 1% L-glutamine and 5% foetal bovine serum (FBS), supplemented with EGF (20ng/ml) (Peprotech), Hydrocortisone (0.5mg/ml) (Sigma Aldrich) and human Insulin (10μg/ml) (Sigma Aldrich). MRC-5 fibroblasts were obtained from the ATCC and maintained in Minimum essential medium (MEM, PAA) containing 10% FBS.

NIH-3T3 mouse fibroblast cells were obtained from the ATCC and cultured in DMEM containing 10% donor calf serum (DCS, Gibco Life Technologies). NIH-3T3 mouse fibroblast cells stably transfected with either wild type murine Met, or the mutated versions of murine Met M1268T and D1246N were a gift from Prof. G. Vande Woude[203]. They were cultured in DMEM containing 10%

donor calf serum (DCS, Gibco Life Technologies)[159, 203]. NIH-3T3 mouse fibroblast cells stably transfected with either human Wt Met or the mutated versions of Met M1268T and M1268T/N1358H were cultured in DMEM containing 10% donor calf serum (DCS, Gibco Life Technologies) and 800μg/ml G418 (Sigma Aldrich).

The β1-integrin-deficient GD25 cell line (GD25), GD25 cells expressing wild-type β1A (β1A) or mutant β1A (β1A-YYFF)[263] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) (Sigma Aldrich), and 2 mM l-glutamine, with the addition of puromycin (5 μg/ml)(Sigma Aldrich) for the β1A and β1A-YYFF cells.

T-REx™-293 cell line stably transfected with Met-GFP ("Met-GFP cells") were maintained in DMEM containing 10% FBS Penicillin/Streptomycin and 5 mg/ml Blasticidin (Invitrogen Life Technologies) with the addition of 400 μg/ml of Zeocin (Invitrogen Life Technologies). Expression of Met-GFP was induced by treating the stable cell line with indicated doses of tetracycline for indicated times (for most experiments, 0.1 μg/ml for 16 hours).

All cells were cultured in an humidified atmosphere of 8% CO_2 at 37^{o}C and sub-cultured twice a week.

2) Patients and tissue samples

TMA

Tissue microarrays (TMAs) were constructed by Dr. James Hulit and Dr. Colan Ho-Yen from 153 cases of invasive breast cancer, 47 cases of *in-situ* breast cancer (DCIS) and 5 cases of normal breast tissue in triplicate (3 x 0.6mm cores) from each formalin fixed, paraffin embedded tumour block obtained from the Barts Cancer Institute tissue bank, kindly provided by Professor Louise Jones. TMA sections were cut at a thickness of 4μm and placed on coated slides.

3) Reagents

a) Antibodies

Antibodies for Western blotting:

25H2 anti-Met (1/1000), rabbit polyclonal phospho-p44/42 MAP kinase (Thr 202/Tyr 204)(1/1000), rabbit polyclonal phospho-Met (Tyr 1349)(1/1000), phospho-Met (Tyr 1234-1235)(1/1000), Gab1 (Tyr 307)(1/1000) and phospho-AKT (Ser 473)(1/1000) from Cell Signaling; mouse monoclonal anti-α-tubulin (1/5000) from Sigma Aldrich; rabbit polyclonal anti-human Met intracellular domain (CVD13) from Invitrogen Life Technologies); rb2 (1/1000), c-Cbl

(1/1000), HSC-70 (1/2000), and Met (B2) (1/200) from Santa Cruz Technologies; Clathrin heavy chain(1/1000) and HSP90 \(1/1000) from BD;

HSP90α (1/1000) from Abcam; HSP90β (1/1000) from BIOMOL International;

mouse monoclonal anti-GFP from CR-UK and rat monoclonal anti-mouse anti- β1-integrin, clone MB1.2 (MAB1997) from Millipore.

Secondary anti-goat, anti-rat, anti-mouse and anti-rabbit IgG horseradish peroxidase(HRP)-coupled antibodies (1/1000) were obtained from Amersham.

Antibodies for immunofluorescence:

Goat polyclonal anti-early endosome antigen 1 (EEA1) (1/100) from Santa Cruz Technologies; phospho-tyrosine (4G10) (1/100) from Upstate, anti-human Met from R&D (1/100); mouse anti-human Met (1/100) from Novacastra; rat monoclonal anti-mouse anti- β1-integrin, clone MB1.2 (MAB1997) from Millipore; rat monoclonal anti- β1-integrin in active conformation, clone 9EG7 from BD Biosciences; mouse monoclonal anti-human anti- β1-integrin, clone DF7 from Biomol International and rat polyclonal anti- β1-integrin (AIIB2) from the Developmental Studies Hybridoma Bank.

Alexa 488 labelled donkey anti-goat or anti-mouse IgG secondary antibodies (1/500) were obtained from Molecular Probes. Cy3-labelled and Cy5 labelled donkey anti-mouse or anti-rabbit IgG secondary antibodies (1/1000) were obtained from Jackson Immunoresearch.

Antibodies for flow cytometry:

Anti-human Met (1/50) was obtaind from R&D. Alexa-488 donkey anti-goat IgG (1/250) from Molecular Probes was used as a secondary antibody.

b) Inhibitors and growth factors

The Met inhibitor SU11274, the VEGFR inhibitor SU1498 and the EGFR inhibitor AG490 were obtained from Calbiochem and were used at 2μM, 2μM and 50μM respectively. PHA-665752 and PF-2341066 were kindly given to us by Dr. Christensen (Pfizer, La Jolla) and were used at 100nM and 200nM respectively. Dynasore, the FGFR inhibitor PD17074 and cycloheximide were purchased from Sigma Aldrich and used at 80mM, 25nM and 50μg/ml respectively. In experiments where Dynasore treatment was required, it was necessary for all cells to be starved in 0% serum for 24 hours. Dynole 34-2 was used at 50μM and provided as a gift by P.J. Robinson (Children's Medical Research Institute Australia). The VEGFR antibody DC101 was a kind gift from Dan Hicklin, ImClone Systems, Inc. and was used at 1.25μg/ml. 17-AAG obtained from Calbiochem was used at 100nM/150nM/200nM as indicated. Alexa-546-conjugated phalloidin (used at 1/250) and Cy3-conjugated transferrin (used at 1/500) were purchased from Molecular Probes. Tetracycline was used at 0.1 μg/ml for the indicated times (16 hours for most experiments) and was obtained from Sigma Aldrich. Mounting media containing DAPI was obtained from BD Pharmaceutics. Purified human recombinant HGF (R&D Systems) was used at 50ng/ml unless otherwise stated.

c) Plasmids

hWt Met construct

The human Met Wt construct has a V5/histidine tag and is within the pRK5neo plasmid obtained from Dr. M. Kong-Beltran (Genentech, San Fransico)[201]. hM1268T and hM1268T/N1358H were produced using the Quick Change II XL mutagenesis kit (Stratagene) by Dr. Véronique Calleja and checked by full sequencing by Dr. Ludovic Menard.

d) RNAi and shRNA

Below are the sequences of RNAi/shRNA used. The AllStars Neg. Control siRNA (20 nmol) (no sequence provided) was obtained from Qiagen. Lentiviral transfection of shCHC was performed by Carine Joffre.

Target	Type	Company	Sequence number	Sequence
Met	RNAi	Dharmacon (siGenome Smart Pool)	1	GGACUUUGCUGGACAAUGA
			2	GAACAGCAGCUAAAUAUA
			3	GGGAAGAAGUGUUUAAUAU
			4	CCAGAGACAUGUACGAUAA
HSP90 alpha	RNAi	Dharmacon (siGenome Smart Pool)	1	CAUCAACACUUUCUAUUCA
			2	GAAACAUUCGCAGUUCAUA
			3	UGACUGAGCCUAUUGACGA
			4	GAUAUGAGAGCCUGACGGA
HSP90 beta	RNAi	Dharmacon (siGenome Smart Pool)	1	GAGCUUAACUAACGAUUGG
			2	GGAAUUAAUCCCUGAGUAU
			3	GAACCAAUGGGUCGUGGAA
			4	CAUCGGACGCUCUGGAUAA
STIP1	RNAi	Dharmacon	2	CCAAGGAACUGGACCCUAC
			3	GCGCUUGGCUUAUAUCAAC
Clathrin Heavy Chain	shRNA	Sigma Aldrich	1	CCGGGCGAACATCAATAGATGCTTACTCG
				AGTAAGCATCTATTGATGTTCGCTTTTTG
	RNAi	Dharmacon	1	CAACUUAGCUGGUGCUGAA

			2	UAGAGGAGCUUAUCAACUA
c-Cbl	RNAi	Dharmacon	1	GAUCUGACCUGCAAUGAUU
Grb2	RNAi	Dharmacon	1	UGAAUGAGCUGGUAGAUUA
			3	CGACGGAGCCGGGAAGUAU
β1-integrin	shRNA	Sigma Aldrich	43	CCGGCCAACCTGTTTACAAGGAATTCTCG AGAATTCCTTGTAAACAGGTTGGTTTTTG
			44	CCGGCCAAGTTTCAAGGGCCAACTTCTCTC GAGAAGTTGGCCCTTGAAACTTGGTTTTTG
			45	CCGGGCCATTACTATGATTATCCTTCTCG AGAAGGATAATCATAGTAATGGCTTTTTG
			46	CCGGCCCGACATCATCCCAATTGTACTCG AGTACAATTGGGATGATGTCGGGTTTTTG
			47	CCGGGCACGATGTGATGATTTAGAACTCG AGTTCTAAATCATCACATCGTGCTTTTTG
	RNAi	Dharmacon	3	GCACAGAUCCCAAGUUUCA
	RNAi	Qiagen	-	AAGAAGGCTCGAGAGTCCTAT

4) Western Blot Analysis

Stimulation experiments

Breast cancer cell lines and GD25 cells were plated in 6 well plates at $2.5x10^5$ cells/well for 24 hours and then starved in serum-free medium for 24 hours.

NIH3T3 cells were plated in 6-well plates at 10^5 cells/well for 24 hours. However, when Met degradation was being investigated, NIH3T3 cells were plated in 6-well plates at $4x10^4$ cells/well for 48 hours. Cells were treated with 17-AAG (at the indicated dose) 16 hours prior to the experiment and cycloheximide (1/100) 2 hours prior to the experiment.

On the day of the experiment, where required cells were pre-treated with inhibitors for 30 minutes and then stimulated in serum-free medium with 50 ng/ml of HGF for the times indicated.

Cell lysis

Cells were put on ice, washed with cold PBS and incubated in RIPA lysis buffer (Upstate) containing 1mM sodium orthovanadate (Sigma Aldrich), 1mM sodium fluoride (Sigma Aldrich), and protease inhibitor cocktail (1/100, Calbiochem) for 10 minutes. Cells were then harvested and placed on a rotating wheel for 20 minutes,at 4^{o}C, after which the lysates were centrifuged at 13000g for 10 minutes at 4^{o}C. The pellet was discarded and the Bio-Rad D_c Protein Assay Kit was used as described by the manufacturer (Bio-Rad Laboratories) to determine the protein concentration of each sample.

Western blot

An equal amount of protein (as determined by the Bio-Rad D_c Protein Assay Kit) was diluted in 4x sample buffer (Invitrogen Life Technologies) containing DTT (Dithiothreitol), except when Western blotting under non-reducing conditions (anti-mouse anti- β1-integrin MAB1997), in which case sample buffer was used without DTT. The proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were loaded into pre-cast gels (4-12% Bis-Tris Gel, Invitrogen Life Technologies) and run for 1.5 hours at 150 V. Rainbow Molecular Weight Marker (GE Amersham) was loaded into one lane to monitor the electrophoresis progress. Proteins were then transferred to Hybond nitrocellulose membranes (Schleider & Schuell) using a wet transfer system at 35 V over 3 hours. Membranes were incubated with TBS containing 3% BSA (Sigma Aldrich) and 0.1% Tween (Sigma Aldrich) for 20 minutes, Membranes were then incubated with the indicated antibodies diluted in TBS

containing 3% BSA and 0.1% Tween for a minimum of 5 hours at 4^{o}C. Following three 10 minute washes with TBS containing 0.1% Tween (TBST), Membranes were incubated with horseradish peroxidase-linked secondary antibodies in TBS containing 3% BSA and 0.1% Tween for 1 hour at room temperature. Following three 10 minute washes with TBST, Enhanced Chemoluminescence reagents (Amersham Biosciences) were used to visualise the protein. Chemiluminescence films (Amersham Biosciences) were exposed and developed.

Densitometry

Protein levels were quantified using ImageJ software. All values were normalised to the loading control tubulin or HSC70 or alternatively total protein levels when measuring phosphorylated proteins.

5) Flow Cytometry

Met internalisation assay

Cells were plated in 24 well plates at 5 x 10^4 for 24 hours prior to being stimulated in serum free medium with HGF (100ng/ml) for the indicated times. Cells were immediately placed on ice at the end of the HGF stimulation to prevent further Met internalisation and were kept on ice for the duration of the experiment. Cells were incubated with cold acid wash medium (DMEM and HCL + 1% BSA) for 7 minutes, followed by two washes in cold FACS buffer (PBS +2% SVF). Cells were then incubated at 4^{o}C with goat anti-Met antibody (R&D systems), diluted in FACS buffer, for 45 minutes.

Two controls were used: no primary antibody or a Goat IgG isotype primary antibody. Cells were washed three times in cold FACS buffer and incubated with anti-goat-Alexa-488 secondary antibody for 30 minutes. After which, the cells were washed three times in cold FACS buffer and detached using EDTA diluted in cold FACS buffer at a concentration of 5mM (300μl/well). The analysis took place using a BD FACSCalibur.

6) Immunofluorescence

Immunofluorescence on cells

Cells were plated in 24 well plates onto 13mm glass coverslips. Breast cell lines were plated at $5x10^4$. NIH3T3 cells were plated at $2x10^4$ onto coverslips coated with 0.01% poly-l-lysine (Sigma Aldrich). GD25 and HEK293 cells were plated at $5x10^4$ onto coverslips coated with 0.01% poly-L-lysine (Sigma Aldrich). After 24 hours, or 48 hours if 24 hours of starvation or 16 hours of tetracycline treatment was required, cells were pre-treated with inhibitors where required and stimulated with HGF (50ng/ml) or HGF labelled with alexa-555 (50ng/ml)(HGF*) at indicated time points, and fixed in 4% paraformaldehyde for 10 minutes. Free aldehydes were quenched in NH_4Cl 50mM for 10 minutes. Aspecific sites were blocked and the cells permeabilised in PBS containing 2% BSA and 0.1% Triton x 100 for 15 minutes. Cells were then incubated the primary antibody (dilution 1:100) in PBS-2% BSA for 30 minutes. Cells were washed 3 times in PBS prior to incubation in the secondary antibody (dilution 1:500) in PBS-2% BSA for 30 minutes. After three washes in PBS and one wash in dH_2O, coverslips were

mounted on glass slides with Prolong Gold antifade reagent (Invitrogen), which contains DAPI. When the 9EG7 antibody was used, 5mM EGTA (Ethylene glycol tetraacetic acid) and 2mM $MgCl^2$ were added to the PBS.

Immunofluorescence on the paraffin embedded sections

Following de-waxing in xylene and rehydration in decreasing concentrations of ethanol, sections underwent antigen retrieval in citrate buffer heated in the microwave for 20 minutes. Aspecific sites were blocked in PBS containing 0.5% BSA and 0.5% donkey serum for 30 minutes. The sections were then permeabilised in 0.1% Triton. Sections were stained with a mouse anti-human Met (1/100,Novacastra) and rabbit EEA1 (1/100) antibodies overnight, which were then detected using secondary antibodies (1/500) labelled with the appropriate fluorohrome, incubated for 2 hours. Sections were rehydrated and then mounted with Prolong Gold antifade reagent (Invitrogen Life Technologies).

Confocal microscopy

A confocal laser scanning microscope LSM510 (Carl Zeiss) with a 63x oil immersion objective, was used to analyse the coverslips. Alexa 488 was excited with the 488-nm line of an argon laser, Cy3 was excited with a 543-nm HeNe laser and Cy5 was excited with a 633-nm HeNe laser. Each image represents a single section 0.7μM thick.

Quantification of cells lacking stress fibres and cells with Rac at the plasma membrane were carried out on a minimum of 100 cells per condition per experiment.

7) Cell Transfection

Amaxa® Nucleofactor® technology transfection

Both DNA and RNAi transfection of NIH3T3 cells were performed using an electroporation method using Amaxa® Nucleofactor® technology following the manufacturer's instructions (Lonza). The nucleofection kit used was R using nucleofection program U-30. Cells were transfected when 70% confluent and plated for experiments directly following transfection. Cells were harvested or subjected to experimental procedures 72 hours after transfection unless otherwise stated.

Lentiviral transduction

Transductions to obtain a stable knockdown of protein expression were carried out using MISSION™ TRC Lentiviral Transduction Particles (Sigma Aldrich) in a category II containment laboratory. 3000 cells were plated per well of a 96-well plate and 24 hours later lentiviral particles were added to the cells (which were no more than 70% confluent) in fresh media at a multiplicity of infection (MOI) 5 and 10. The following day, the medium was removed, the cells were washed gently, to remove viral particles, and fresh medium was added containing the selection agent puromycin (at 2µg/ml, the concentration previously determined to induce cell death in NIH3T3 cells over 3-5 days). Once resistant cells had expanded sufficiently, Western blots were performed on cell lysates to determine knockdown efficiency.

8) Cell Proliferation Assay

GD25 cells were plated in 24 well plates at 10^4 cells/well on day 0 and incubated in complete medium with or without HGF (10ng/ml). Two wells of cells were trypsinised and counted (twice per well) using a fast read haemocytometer after 24 (day 1), 72 (day 3) and 144 hours (day 6).

9) Wound Healing Assay

Cells were plated in 6 well plates at 2.5 x 10^5 for 24 hours and then incubated in serum-free medium for 24 hours. Prior to induction of the wound, cells were pre-treated with DMSO, SU11274 or Dynasore for 30 minutes. After which cells were scraped away (wounded) using a standard 100μl Gilson pipette tip, washed with serum-free medium and incubated with or without HGF. Pictures were taken of the wounds at time 0 and 17-24 hours later (as indicated). Quantification of the extent of wound closure was performed by measuring the wound area at 24 hours compared to 0 hours, using Adobe Photoshop.

10) Transwell Migration Assay

NiH3T3 cells were starved in DMEM containing 1% DCS 24 hours prior to the assay.

Transwells (Corning) were removed from the plates and the underside of each membrane (containing 8μM pores) was coated with fibronectin (Sigma

Aldrich), diluted in PBS 1:100. After 30 minutes, the fibronectin was removed and the membranes were left to dry for 1 hour at room temperature. 600µl of complete medium was placed into the lower chamber below the membrane. HGF (50ng/ml) was added to this lower chamber where required. $2x10^4$ cells were suspended in serum free medium and put into each Transwell, above the membrane. Where inhibitors were used, cells were pretreated for 30 minutes and incubated with the inhibitor. NIH3T3, human breast cancer cell lines and GD25 cells were left for 2, 4 or 6 hours respectively. Media was then removed from each transwell and cells on top of the membrane were removed with cotton buds. Cells were fixed in 4% paraformaldehyde for 10 minutes, washed in PBS and the nuclei were then stained with haematoxylin for 20 minutes. After washing in PBS, the membranes were removed using a scalpel and mounted onto glass slides using an aqueous mounting medium (Aquatex, VWR). Cells in ten random fields were counted per membrane at a magnification of 20x using a Zeiss Axiophot microscope.

11) Biotin Internalisation Assay

In this experiment there are three conditions:

Total surface: Surface of cells undergo biotinylation but this is not cleaved, allowing the total amount of Met on the cell surface to be analysed.

'0' minutes: Surface of cells undergo biotinylation and biotin cleavage, allowing the efficiency of the cleavage to be assessed.

'15' minutes: Surface of cells undergo biotinylation, surface proteins are then allowed to internalise for 15 minutes at 37 ^{o}C and then biotin is cleaved from remaining proteins on the cell surface.

Plated cells were treated with cycloheximide 12 hours prior to the assay to inhibit protein synthesis.

Cells were placed on ice and washed twice with cold PBS, they were then incubated with cold Biotin (0.2mg/ml) on a rotator for 45 minutes at 4^{o}C. Following one wash with cold PBS the plates for which internailisation was to be measured had warm medium added and they were placed in the incubator at 37 ^{o}C for 15 minutes. The Total surface and '0' timepoints remained on ice during this time at 4 ^{o}C. Following this, the cells were washed twice with PBS and the biotin was cleaved from the cell surface of the 0 and 15 minutes plates, using 8 ml freshly prepared 180mM MesNa cold reagent (Cat 63705, Sigma Aldrich), on a moving platform for 20 minutes at 4 ^{o}C. The residual MesNa was then quenched by adding 1.6 ml freshly prepared 180mM Iodoacetamide (Cat i1149, Sigma Aldrich) to each plate and leaving them on the moving platform for a further 10 minutes at 4^{o}C. Following two washes with PBS, the cells were then lysed in RIPA buffer. Meanwhile, 100ul of streptavidin-coated beads (Cat 16-126, Upstate) per sample were washed. Equal amounts of protein in an equal amount of lysis buffer were then added to the beads and they were incubated together on a rotating wheel at 4^{o}C for 2 hours. Samples were then centrifuged and the beads were washed 3 times with lysis buffer at 4 ^{o}C, after which 25μl of warm sample buffer was added to the beads. After 10 minutes at 95 ^{o}C the samples were analysed by Western blot analysis.

The percentage of internalised Met was calculated using the following formula:

Internalised Met = [(Met level after incubation at 37 ^{o}C)-(Met level at 0 minutes)]/(total surface Met x 100).

12) Soft Agar Assay

0.3% agarose (type IX-A, Sigma Aldrich)(in PBS) was mixed with 5ml complete media per condition, which was subsequently filtered through a 0.22μm filter (Millex®, Millipore) and kept in a water bath at 37°C until needed. Cells were passed through a 21g syringe, to ensure single cell suspension, and then counted. 500 cells were placed into the bottom of each tube, on top of which 5ml of the media mixed with 0.3% agarose was then poured gently. The cells were gently resuspended throughout the medium-agar mixture and the tubes were left to solidify on ice for approximately 20 minutes. 1ml of complete medium was placed on top of the gels prior to them being placed at 37 ^{o}C and this medium was changed every 2 days.

When inhibitors were used, they were added, once colonies began to form (Day 5 for NIH3T3 cells expressing various forms of Met), to the 1ml medium placed on top of the gels and this was changed every day. The concentration of the inhibitors was calculated so as to apply for the total volume. In experiments with GD25 cells HGF (14 ng/ml) was added or not daily as soon as colonies were observable by eye (day 8). When the experiment was due to be stopped (day 9 for NIH3T3 cells, day 6 when transfected with siRNA, and day 13 for GD25 cells), the medium on top was removed and the gels were poured into wells of a 12 well plate. Photos were then taken of whole wells using a Zeiss, Stemi SV11 microscope. Analysis took place by both

counting the number of colonies and measuring the area of the colonies using ImageJ software.

13) Organotypics

To make the organotypic gels a mixture of 3.5 parts collagen (Collagen 1 rat tail, VWR International), 3.5 parts Matrigel (BD Matrigel Basement Membrane Matrix, VWR International), 1 part 10x DMEM (CR-UK Media Services), 1 part FBS and 1 part MEM containing MRC-5 fibroblasts (5 x 10^5 cells per gel), was made on ice. 1ml of the combined mixture was then added to each well of a 24 well plate for as many gels as required and placed at 37 ^oC for 1 hour to solidify. 1ml of MEM containing 10% FBS was placed on top of each gel and they were left at 37 ^oC overnight. The following day the media was removed from the tops of the gels and 5 x 10^5 of either MDA-MB-231 or MCF-10ADCIS.com cells suspended in 1ml of their normal complete media, were plated onto the top of each gel. The gels were then left overnight at 37 ^oC. On the same day sterile nylon sheets approximately 2cm^2 (one for each gel) were coated with a mixture of 7 parts collagen, 1 part 10x DMEM, 1 part FBS and 1 part MEM + 10% FBS at a neutral pH. The sheets were left at 37 ^oC for 30 minutes, after which a 1% glutaraldehyde solution (in PBS)(Sigma Aldrich) was added to the sheets and left at 4°C for 1 hour. Following 3 washes with PBSA and once in serum containing media the sheets were left in MEM + 10% FCS overnight at 4°C. The following day, one steel grid was placed into each well of a 6 well plate and a gel coated nylon sheet was placed on top (with collagen side facing upwards). Media were

removed from the top of each gel and the gels were then gently removed from the 24 well plate using a sterile spatula and placed onto each of the nylon sheets. 4.85ml of complete media (for the appropriate epithelial cells) were placed into each well so that it reached the under surface of the grid. Any inhibitors used were placed in the gels, with the epithelial cells and in the media placed beneath each gel. Media were replaced every 2 days and the gels were harvested after 14 days of culture. The gels were fixed in 4% paraformaldehyde for 24 hours and then stored in 70% ethanol, prior to being paraffin embedded. Sections of 8μm were cut for analysis. Haematoxylin eosin staining was performed and pictures taken on a Zeiss Axiophot microscope. ImageJ software was used to calculate the Invasion Index as developed by Nystrom et al.[264]:

Invasive Index:

Number of invading particles in the gel X total area of invading particles X average depth of invading particles

14) *In vivo* tumourigenicity assays

4-6 week old female athymic nude mice (CD1 Nu/Nu, Charles River UK) were used in accordance with United Kingdom Coordination Committee on Cancer Research guidelines and Home Office regulations.

Tumour growth assay

A 5 x 10^6/ml suspension of cells in PBS was prepared and 5 x 10^5 cells were injected (100μl of solution) subcutaneously into the right flank of nude mice

by Prof. Ian Hart. Tumours were measured daily using callipers and volumes were calculated using the following formula:

$$\text{length} \times \text{width}^2 \times (\pi/6)$$

Once tumours reached 50mm^3, 100μl of DMSO (control) or the relevant reagent was painted onto the skin over the top of the tumour and the surrounding skin. Once tumours reached 1cm in length, mice were killed humanely and the tumours were dissected. Part of each tumour was fixed in formal saline to be embedded in paraffin and part was snap-frozen for analysis by Western blotting.

Lung colonisation assay

A 5 x 10^6/ml suspension of cells in PBS was prepared and 5 x 10^5 cells were injected (100μl of solution) into the tail vein of nude mice by Prof. Ian Hart. After 21 days, mice were killed and the lungs were weighed and fixed in formal saline for 24 hours after which they were stored in 70% ethanol. Lungs were cut and embedded in paraffin, from which 4μM sections were cut and stained with haematoxylin/eosin. Analysis and image acquisition was undertaken using a Zeiss Axiophot microscope.

15) Statistical Analysis

Student's t-Test was performed, following a two-tailed distribution, paired when comparing the same cell line and un-paired when comparing different cell lines. Met expression in tissue samples was analysed using a Chi-square

with Graphpad Prism 5 software. Probability of <0.05 was considered significant.

RESULTS CHAPTER I

Met endosomal signalling correlates with breast cancer progression

1) Met is phosphorylated by HGF to a similar level in MCF10ADCIS.com and MDA-MB-468 cells

To investigate the role of Met and the mechanism of Met signalling in the progression of breast cancer, two breast cancer cell lines with different invasive phenotypes were studied: MCF10ADCIS.com and MDA-MB-468. The MCF10ADCIS.com cell line is a model of DCIS (ductal carcinoma in situ) adapted from the normal mammary gland cell line MCF-10A[265]. MDA-MB-468 cells represent a more aggressive model of breast cancer with the ability to form lymph node metastases in nude mice[266]. Both of the cell lines used are representative of the basal breast cancer subtype[267, 268],. Furthermore, we found these cell lines have similar Met expression levels (**Figure 1A**), making investigating differences in Met signalling dynamics more straightforward.

Stimulation with recombinant HGF for 15 minutes led to a similar level of Met phosphorylation (Y1349 in the docking site) in each cell line (around 5 fold increase versus basal level, $p<0.05$) and this signal was sustained over a two hour period (**Figure 1B and C**). The activation of Met was shown to be specific as it was inhibited in the presence of 2μM SU11274, a specific small molecule Met inhibitor (**Figure 1B)**. Additionally, I investigated the downstream signalling of Met in these cells. HGF stimulation led to a

significant activation of ERK1/2 and AKT in the MDA-MB-468 cells **(Figure 1D) (see Figure 6B for quantifications)**. However, no significant activation of these pathways could be detected in the MCF10ADCIS.com cells. This probably is because they already are highly phosphorylated in basal conditions (despite serum deprivation conditions for 24h).

I then went on investigating the influence of Met signalling in cellular functions.

2) HGF stimulates wound healing and chemotactic migration of MCF10ADCIS.com and MDA-MB-468 cells

The addition of HGF led to a significant increase in the level of wound healing in both cell lines (2.2 and 2.6 fold versus no HGF in MCF10ADCIS.com and MDA-MB-468 respectively, $p<0.05$) (**Figure 2**). Treatment with SU11274 had no effect on the levels of basal wound healing and, following stimulation with HGF, the level of wound closure was reduced in both cell lines to that observed in the control with no HGF stimulation, indicating that the wound healing observed upon HGF stimulation is specifically a result of Met signalling.

In Transwell migration assays both the MCF10ADCIS.com and the MDA-MB-468 cells had a significant fold increase (1.8 fold and 1.6 fold respectively, $p<0.05$) in the number of cells migrating through the Transwell membranes upon 4 hours of HGF stimulation compared to the unstimulated control (**Figure 3**).

Thus, HGF triggers wound healing and cell migration of both cell lines.

3) Following HGF stimulation, Met is internalised and traffics to early endosomes, where it remains activated

Previous studies in our lab have reported the importance of Met localisation in signalling[62, 157, 159]. To our knowledge, Met endocytosis / trafficking has not been reported in breast cancer cells. My next step was therefore to characterise the trafficking of Met in the two breast cancer cell lines studied here[62, 157].

In order to follow Met trafficking, our lab has fluorescently labelled HGF, HGF-alexa555 (HGF*)[157]. HGF* colocalises strongly with total Met staining and therefore allows us to follow the localisation of HGF-bound Met reliably (**Figure 4A**). In both cell lines, I observed HGF* uptake indicating an internalisation of Met triggered by HGF. At 30 minutes of HGF, Met appears fully internalised and partially colocalises with the early endosomal marker, EEA1.

Interestingly, HGF* also colocalises with phospho-tyrosine (4G10) and some triple colocalisations HGF*/EEA1/4G10 were observed (**Figure 4B**). This indicates that Met remains bound to its ligand and activated within endosomal compartments in the breast cancer cell lines. I therefore wondered if the presence of an activated form of Met on endosomes was a requirement for Met signalling in these cells and thus what would happen if I blocked endocytosis?

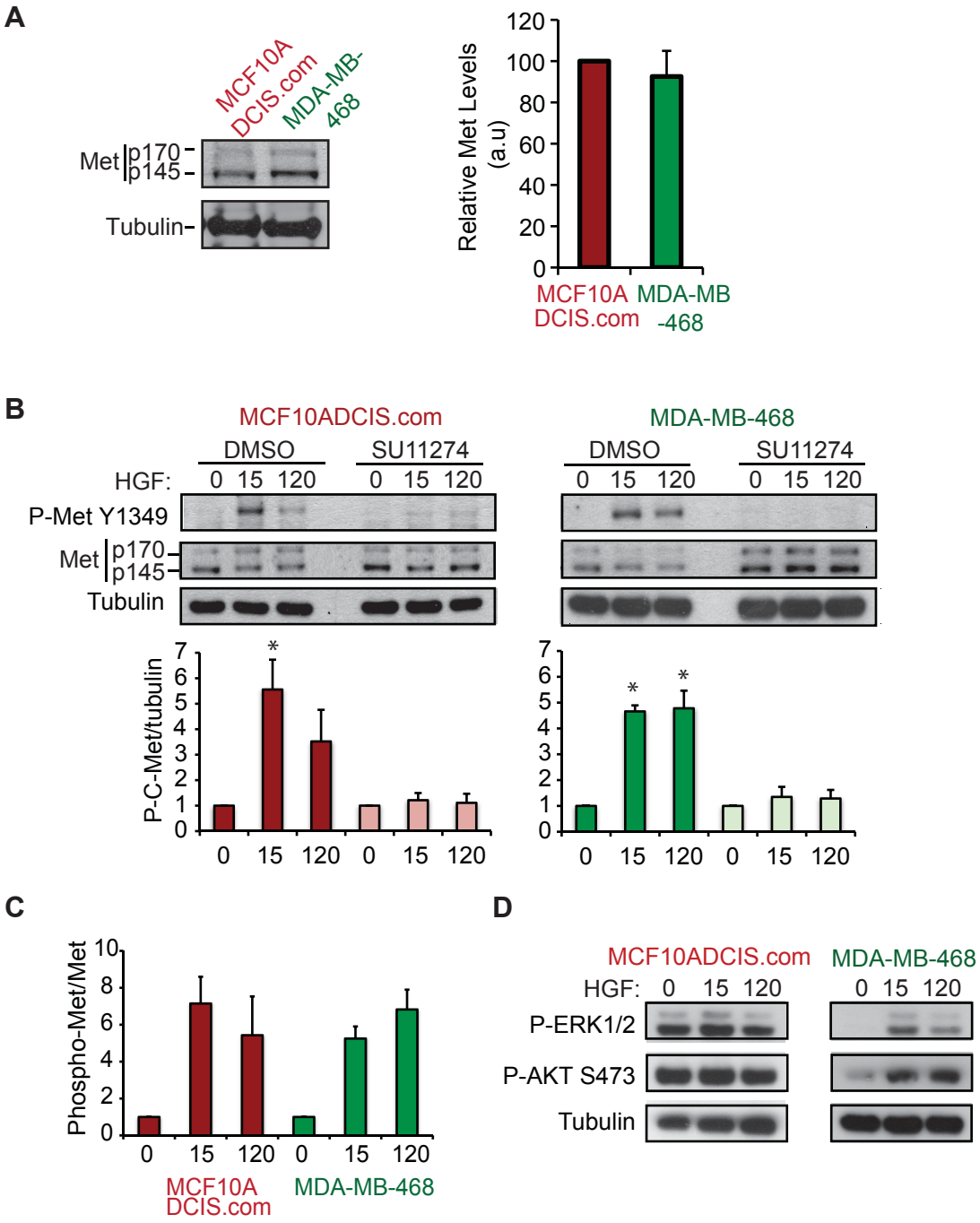


Figure 1: Met is activated by HGF in two basal-like breast cancer cell lines

A-D) Western blots on cell lysates of MCF10ADCIS.com and MDA-MB-468 cells. **A)** Left: Western blot for Met and tubulin. Right: Quantification of Western blots showing Met expression levels normalised on tubulin using ImageJ software (n=3). **B)** Western blots of phospho-Met (Y1349), Met and tubulin upon HGF stimulation (50ng/ml) for 0, 15 and 120 minutes in the presence of DMSO or the Met inhibitor SU11274 (2μM). Bottom panel shows quantification (using ImageJ software) of Met phosphorylation (Y1349) (ratio of phospho-Met/tubulin) (n=3). **C)** Quantification of Met phosphorylation as a ratio of total Met (n=3). **D)** Western blots of phospho-ERK1/2, phospho-AKT and tubulin following stimulation with HGF for 0, 15, 120 minutes (n=3). Data are mean (arbitrary units) ± SEM. *=p<0.05; NS=not significant.

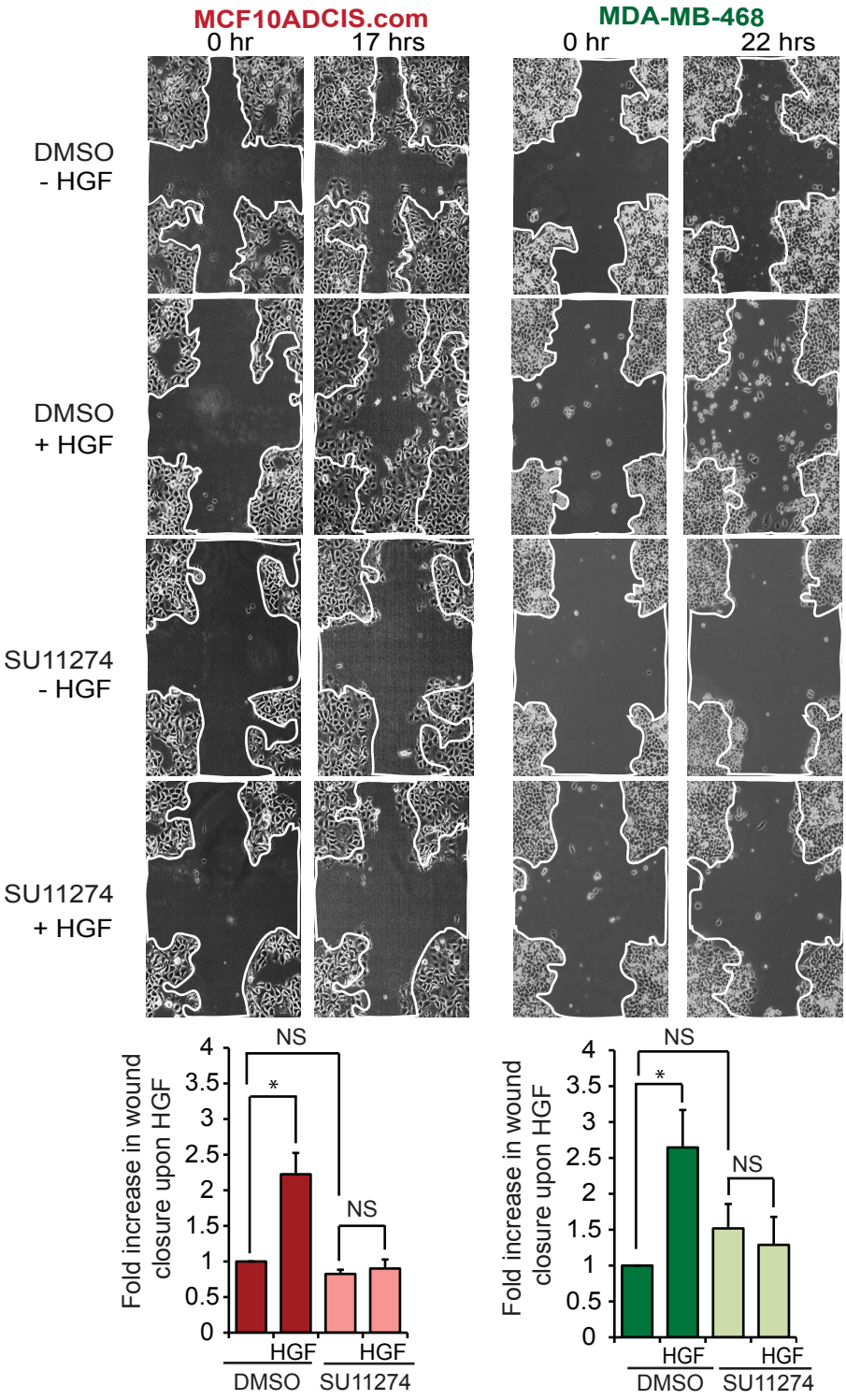


Figure 2: Met activation increases wound healing in two basal-like breast cancer cell lines

Wound healing assays. Wounds were made in confluent layers of MCF10ADCIS.com and MDA-MB-468 cells which were then incubated with/ without HGF (50ng/ml) and treated with DMSO or the Met inhibitor SU11274 (2μm). Pictures of the same wound were taken immediately after the wounds were made (0hr) and 22 hours later. Graphs in the bottom panel are quantifications of mean wound closure expressed as a fold increase upon HGF compared to control. Data are mean (arbitrary units) ± SEM (n=3). *=p<0.05; NS=not significant.

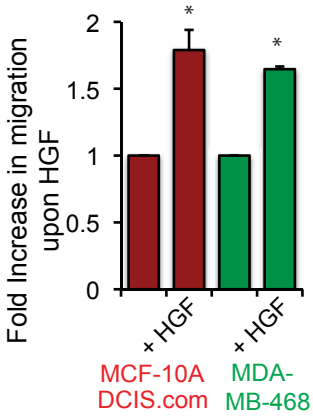


Figure 3: Met activation increases cell migration in two basal-like breast cancer cell lines

Transwell migration assays in which cells were allowed to migrate through a Transwell membrane (pore size = 8μm) for 4 hours. The graph shows the fold increase in cell migration upon HGF in the two breast cancer cell lines. Data are mean (arbitrary units) ± SEM (n=3). *=$p<0.05$.

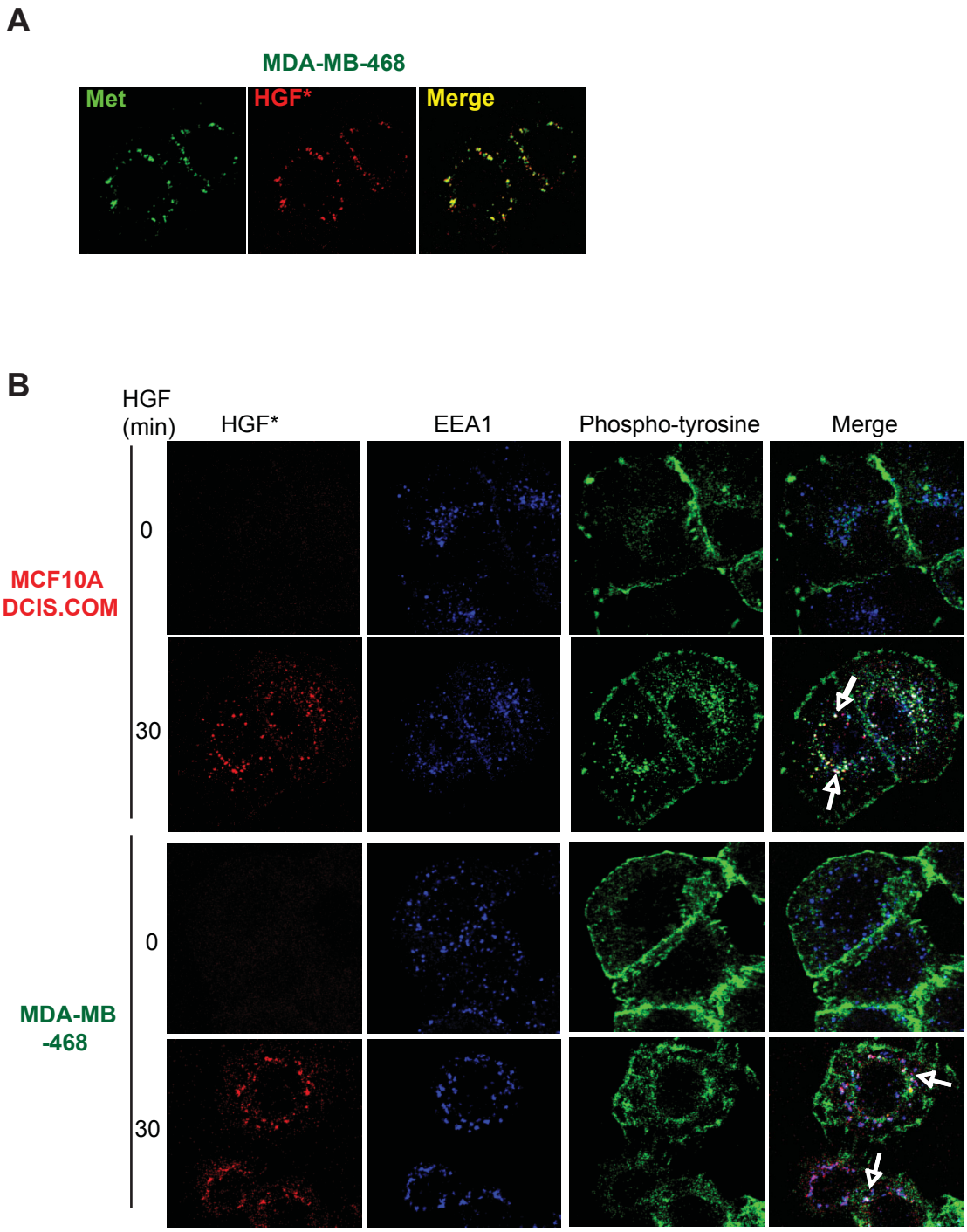


Figure 4: Met is internalised upon HGF stimulation and traffics to early endosomes where it remains activated
A) Immunofluorescence showing colocalisation between HGF (labelled with Alexa 555, "HGF*") and Met in MDA-MB-468 cells. **B)** Immunofluorescence on MCF10ADCIS.com and MDA-MB-468. Cells were stimulated with HGF* (red) for 0 and 30 minutes and then stained with EEA1(blue) and phospho-tyrosine (4G10) (green). White arrows indicate colocalisation between the three colours.

4) The inhibition of endocytosis impairs Met activation in the aggressive MDA-MB-468 cells only

In order to inhibit Met internalisation, I used Dynasore, a cell permeable inhibitor of the activity of the GTPase dynamin[269]. Preincubation of the cells with Dynasore prevented HGF* uptake such that, at 30 minutes**,** more than 90% of HGF* cellular uptake (thus Met endocytosis) was inhibited in both cell lines (**Figure 5A and B)**.

I then investigated the potential influence of the inhibition of endocytosis on Met activation in MCF10ADCIS.com and MDA-MB-468 cells stimulated with HGF (**Figure 6A**). Interestingly the result was very different in the two cell lines. While Met phosphorylation (Y1349) decreased significantly by over 70% at 15 and 120 minutes of HGF stimulation in the MDA-MB-468 cells ($p<0.05$), it remained unchanged in the MCF10ADCIS.com cells (**Figure 6A**).

The effect of Dynasore on HGF stimulated ERK1/2 and AKT activation was then monitored in the MDA-MB-468 cells alone (as it could not be observed in MCF10ADCIS.com cells). Treatment with Dynasore greatly reduced the activation of these signalling molecules, which underwent a 49.5% and a 61% reduction in ERK1/2 phosphorylation ($p<0.05$) and a 48% and a 37% reduction in AKT phosphorylation ($p<0.05$) at 15 and 120 minutes of HGF stimulation respectively (**Figure 6B**).

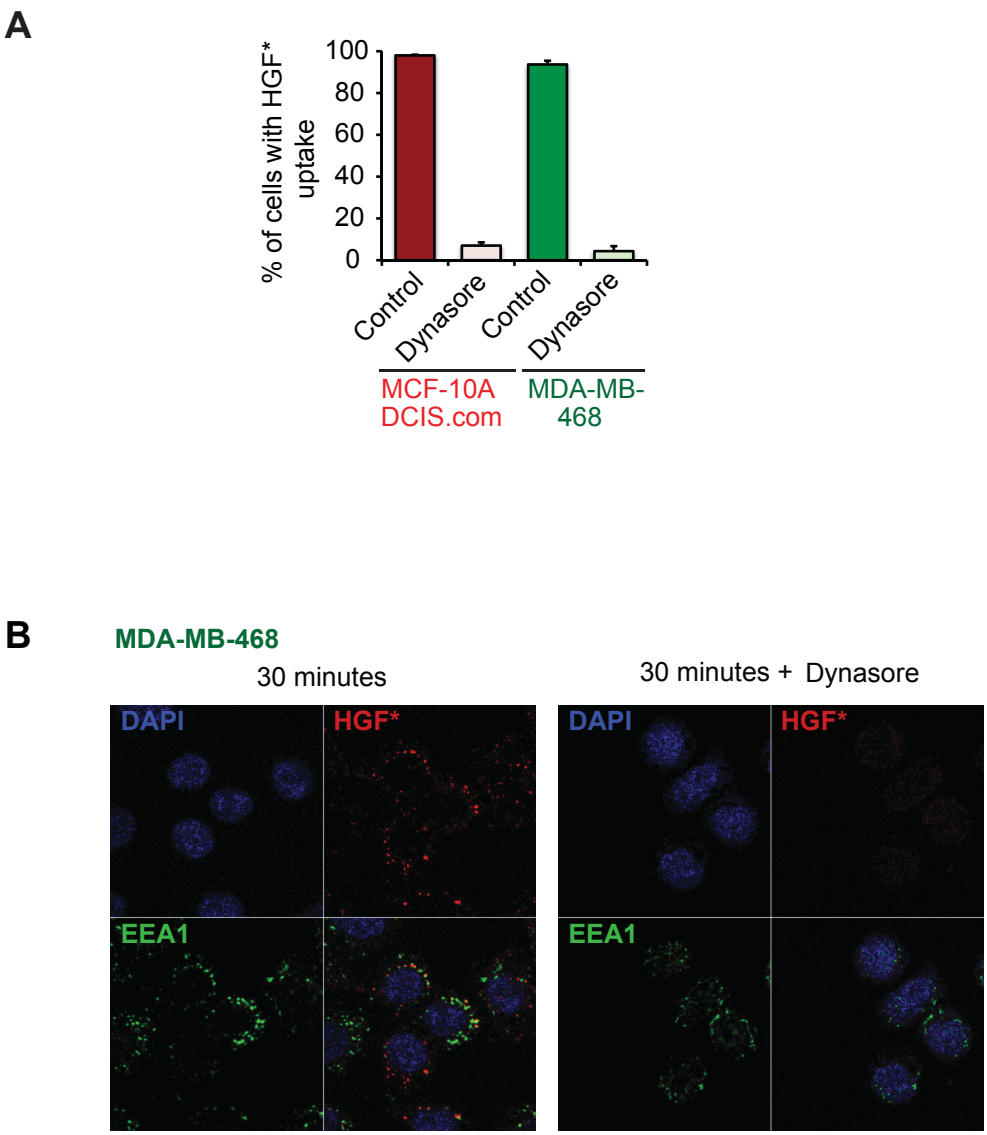


Figure 5: Dynasore blocks Met internalisation
A) MCF10ADCIS.com and MDA-MB-468 cells were treated with HGF* for 30 minutes +/- treatment with Dynasore. Quantification of HGF* uptake was performed by counting 100 cells per experiment, Data are mean (arbitrary units) ± SEM (n=3). **B)** Immunofluorescence showing uptake of HGF* in MDA-MB-468 cells. Cells were also stained with DAPI (blue) and EEA1 (green).

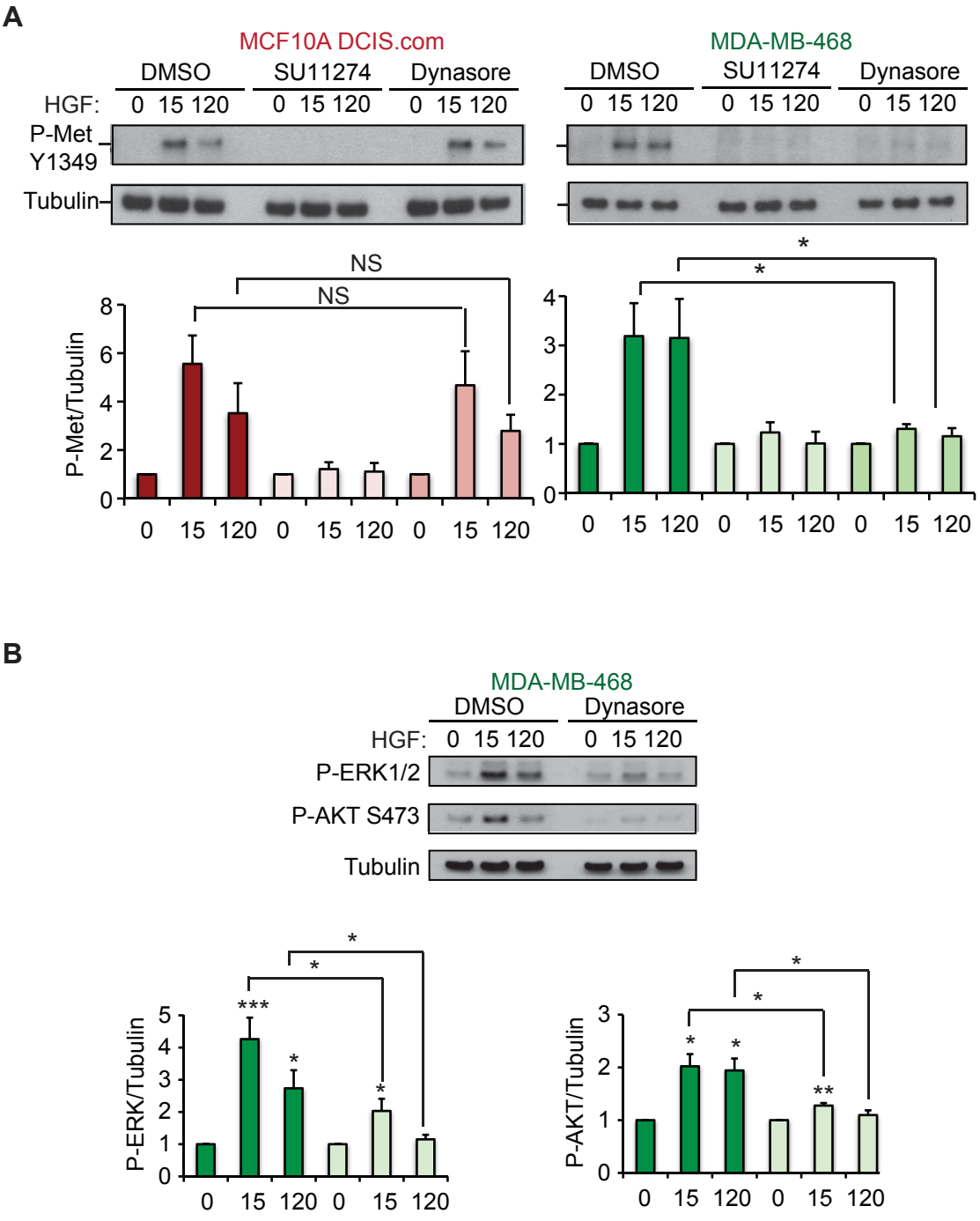


Figure 6: Endocytosis is required for the full activation of Met as well as ERK and AKT in the aggressive MDA-MB-468 cells

AB) Western blots on indicated cell lysates following stimulation with HGF for 15 and 120 minutes +/- Dynasore. Bottom panels are quantification of Western blots using ImageJ software. **A)** Western blot on MCF10ADCIS.com and MDA-MB-468 cell lysates of phospho-Met (Y1349) and the loading control tubulin. Data are mean (arbitrary units) ± SEM (n=3). **B)** Western blot on MDA-MB-468 cells of phospho-ERK 1/2, phospho-AKT and the loading control tubulin. Data are mean (arbitrary units) ± SEM (n=3). *=p<0.05; **=p<0.01; ***=p<0.001; NS=not significant.

These results suggest that there could be a correlation between the aggressiveness of breast cancer cells and the dependence on an endosomal location of Met for its optimal activation.

5) The inhibition of endocytosis impairs the HGF dependent migration of the aggressive MDA-MB-468 cells only

I then repeated the Transwell migration assays in the presence of Dynasore or DMSO. A significant reduction of nearly 60% ($p<0.001$) of HGF dependent migration was observed for MDA-MB-468, while only a small, but non-significant, reduction occurred for the MCF10ADCIS.com cells **(Figure 7)**, even though they have similar levels of cell migration upon HGF stimulation **(Figure 2 and 3)**. Importantly, no reduction in the basal migration was observed following treatment with Dynasore alone (**Figure 7**).

Thus, Met endosomal signalling appears to be required for the full migration of the more aggressive cells, but not for similar behaviour of the pre-invasive MCF10ADCIS.com cells. However Met appears to internalise and be activated on endosomes in both cell lines (**Figure 4B**). We wondered whether a potential difference in the rate of Met internalisation between the two cell lines could occur and explain this result.

6) Met internalises faster in the more aggressive cell line MDA-MB-468

I performed a flow cytometry internalisation assay (see Methods) to quantify Met reduction at the plasma membrane (and thus internalisation) upon time of HGF stimulation. This assay confirmed the immunofluorescence results

(Figure 4B) that Met gets internalised in both cell lines **(Figure 8A)**. Interestingly however, the internalisation rate differed between the two cell lines, with significantly more Met internalised in the MDA-MB-468 (49%) cells as compared to MCF10ADCIS.com cells (34%, $p<0.05$) following 15 minutes of HGF stimulation. Nevertheless, after 60 minutes of HGF an equal amount (50%) of total Met had been internalised in both cell lines (**Figure 8A**). This suggests that Met internalises faster initially in the MDA-MB-468 cells, while it does so at a steadier rate in the MCF10ADCIS.com cells such that the total amount internalised is similar after 60 minutes of HGF. These results were confirmed by immunofluorescence, performed by Dr. Ludovic Menard, a post-doc in the lab. Thus, at 15 minutes, although HGF* was detected in EEA1 positive endosomes in the MDA-MB-468 cells, it mostly was observed at the plasma membrane in the MCF10ADCIS.com cells. It was detected on endosomes at later times (30 and 120 minutes) (**Figure 8B**).

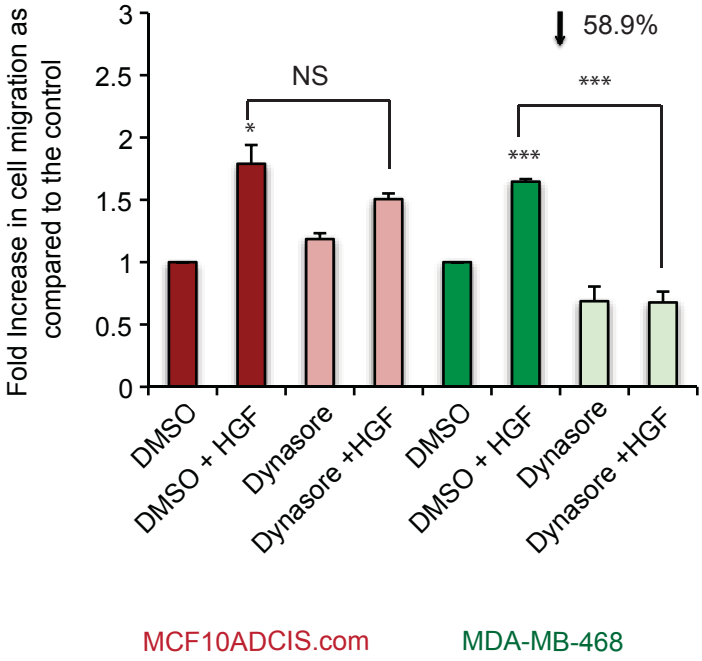


Figure 7: Inhibition of endocytosis reduces Met dependent cell migration of the aggressive MDA-MB-468 cells
The graph shows the fold increase in the number of MCF10ADCIS.com and MDA-MB-468 cells that migrated through Transwell membranes over a period of 4 hours following treatment with DMSO or Dynasore (80μM) and, where mentioned, stimulated with HGF (50ng/ml). Data are mean (arbitrary units) ± SEM (n=3). Percentage decrease in migration is stated. *=p<0.05; ***=p<0.001; NS=not significant.

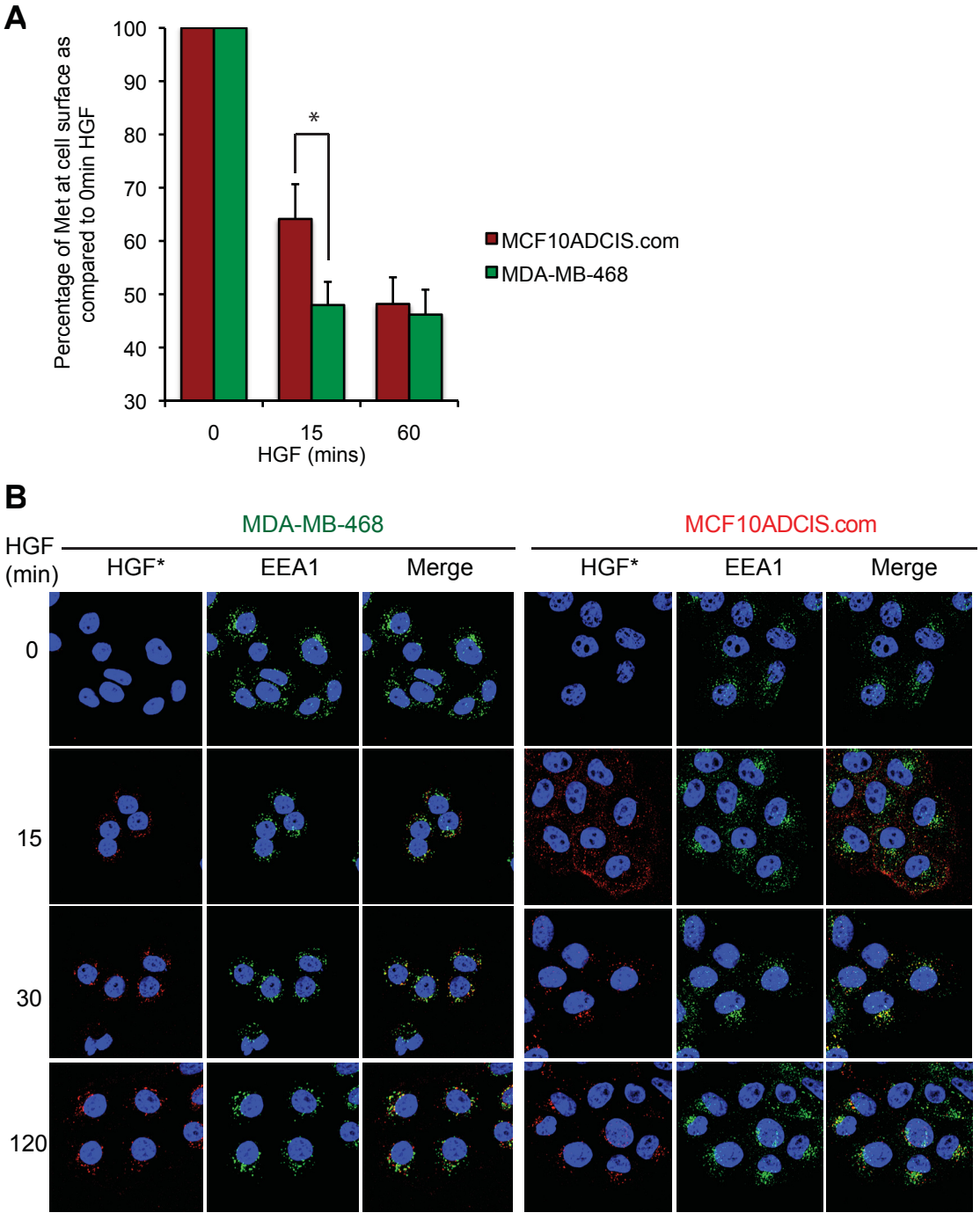


Figure 8: Met traffics faster in the more aggressive MDA-MB-468 cells
A) FACS internalisation assay measuring the amount of Met at the cell surface over 0, 15 and 60 minutes of HGF stimulation (100ng/ml) using FACS Calibur in MCF10ADCIS.com and MDA-MB-468 cells. Data are mean (arbitrary units) ± SEM (MCF10ADCIS.com (n=7) and MDA-MB-468 (n=9)). B) Immunofluorescence on MCF10ADCIS.com and MDA-MB-468 cells. Cells were stimulated with HGF* (red) for 0, 15, 30 and 120 minutes. The cells were then stained with EEA1(green) and DAPI (blue).

These data, showing a difference in the rate of Met internalisation between the two breast cancer cell lines, suggest that the MCF10ADCIS.com cells are less sensitive to Dynasore, in terms of Met activation and Met dependent cell migration, due to the slower internalisation rate of Met. As a consequence, in these cells, a higher proportion of Met may signal from the plasma membrane than in the MDA-MB-468 cells. This is evident at 15 minutes when more Met remains at the plasma membrane in MCF10ADCIS.com cells. However, at 120 minutes this may also be the case, since by FACS I have observed that 47% of Met is still at the plasma membrane in both cell lines (data not shown), so it is possible that the majority of Met signalling is occurring at the plasma membrane in MCF10ADCIS.com cells and on endosomes in MDA-MB-468 cells at this time.

7) Only the more aggressive MDA-MB-468 cells invade upon Met activation and Met is observed on endosomes in aggressive cells.

HGF triggers a similar migration of the MCF10ADCIS.com and MDA-MB-468 cells **(Figure 2 and 3)**. We therefore hypothesised that possibly Met endosomal signalling is important for cell invasion. I investigated the invasive ability of the two cell lines upon Met activation. In order to imitate physiological conditions, the cancer cells were added to a 3D organotypic invasion assay[270] containing an extracellular matrix embedded with fibroblasts[264], in this case MRC-5 fibroblasts that are known to secrete HGF[271]. It was first confirmed that MRC5 conditioned medium activates Met in both cell lines. Indeed MRC5 conditioned medium led to a strong phosphorylation of Met that was inhibited by treatment with SU11274 (**Figure**

9A). It was further verified that cells would migrate across Transwell membranes towards MRC5 cells, cultured in the lower well (**Figure 9BC**). Importantly, SU11274 strongly inhibited the migration (**Figure 9C**) while inhibitors against other RTKs, such as EGFR, VEGFR and FGFR, had no effect (**Figure 9D**), indicating that an important part of the cell migration towards MRC-5 conditioned media is due to Met activity.

Organotypic cultures were then set up consisting of a synthetic extracellular matrix containing MRC-5 fibroblasts (**Figure 9E**). After 10 days of incubation the gels were analysed and an invasive index, as developed by Nystrom et al., was calculated[264]. In organotypic gels with no fibroblasts there was no invasion of the MCF10ADCIS.com cells and a very small, barely discernible, amount of invasion of the MDA-MB-468 cells **(Figure 10AB**). The gels containing MRC-5 cells showed a substantial increase in invasion of the MDA-MB-468 cells (invasive index of 7.73), which was reduced significantly by treatment with SU11274 (invasive index of 1.96, $p<0.05$). MCF10ADCIS.com cells only had a very small non-significant amount of invasion into the gels as compared to the MDA-MB-468 (invasive index of 0.37) cells when MRC-5 cells were present.

Thus, upon HGF/MRC5 stimulation, MCF10ADCIS.com cells are poorly invasive while MDA-MB-468 cells are highly invasive. Initially these results do not seem surprising since MCF10ADCIS.com cells are a model for the pre-invasive ductal carcinoma in situ (DCIS) stage of breast cancer, while MDA-MB-468 cells have a more aggressive phenotype. Conversely though,

Met is activated in both cell lines to a similar degree upon exposure to HGF (**Figure 1A**). In fact, the major differences I have observed between the two cell lines is the sensitivity to Dynasore for Met phosphorylation, Met dependent migration and the rate of Met internalisation.

Interestingly, using immunofluorescence analysis, I observed in the 3D organotypic sections, that Met was present in early endosomes in the individual invading MDA-MB-468 cells (**Figure 11)**. Perhaps the difference in their ability to invade ECM could be due to the localisation of activated Met on endosomes for a longer period of time in the MDA-MB-468 cells. Thus there might be a correlation between Met signalling from the endosome and its ability to trigger cell invasion.

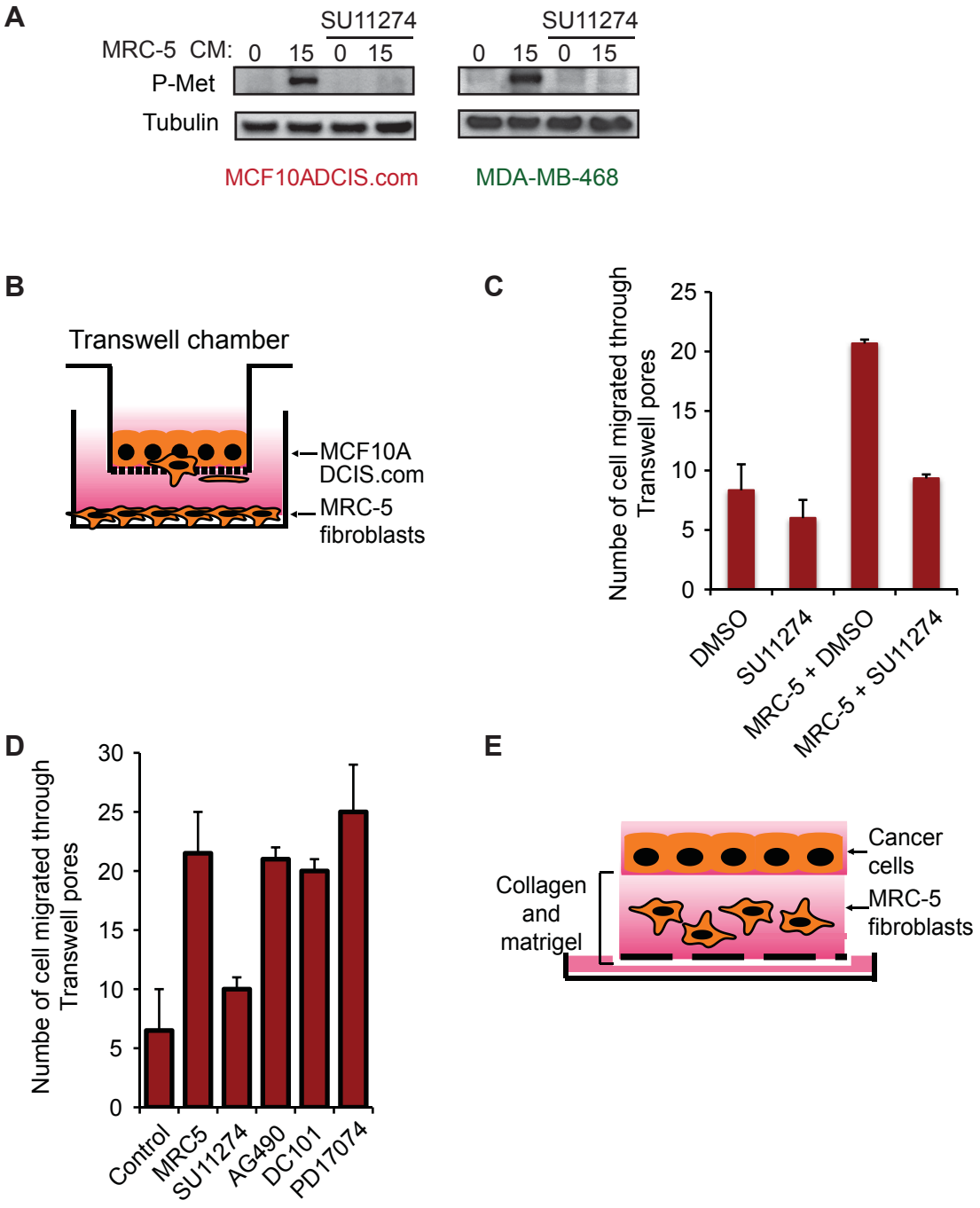


Figure 9: MRC-5 fiboblasts activate Met and breast cancer cell migration

A) Western blot for phospho-Met and tubulin on MCF10ADCIS.com and MDA-MB-468 cell lysates stimulated with MRC-5 conditioned media following treatment with DMSO or SU11274 (2μM). **B)** Diagram of the Transwell migration assay used in **CD). C)** Transwell migration assay with MCF10ADCIS.com cells +/- HGF (50ng/ml) or MRC-5 fibroblasts and treated with DMSO or SU11274 (2μM) (n =1, in triplicate). **D)** Transwell migration assay with MCF10ADCIS.com cells +/- MRC-5 fibroblasts following treatment with DMSO; SU11274 (2μM); EGFR inhibitor, AG490 (50μM); VEGFR antibody, DC101 (1.25μg/ml) and FGFR inhibitor, PD17074 (80mM) (n=1 in duplicate). **E)** Diagram of organotypic cultures.

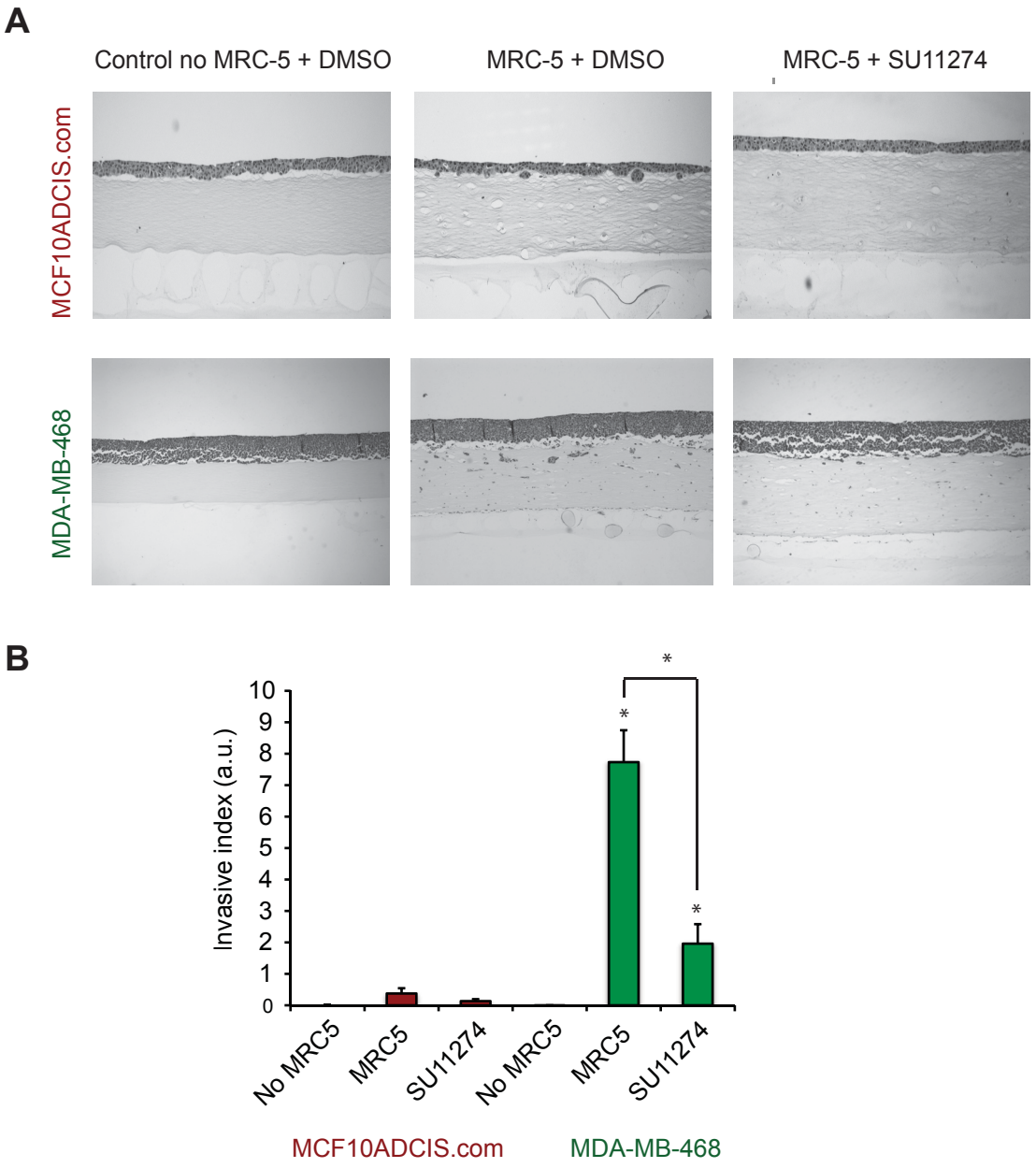


Figure 10: Met activation induces cell invasion of the MDA-MB-468 cells
A) Representative pictures of organotypic cultures without MRC-5 or with MRC-5 fibroblasts in the presence of DMSO or the Met inhibitor SU11274 (2uM). B) Quantification of the level of invasion into organotypic cultures using the invasive index as developed by Nystrom et al. Ten fields analysed per gel (n=3 gels/condition). Data are mean (arbitrary units) ± SEM.

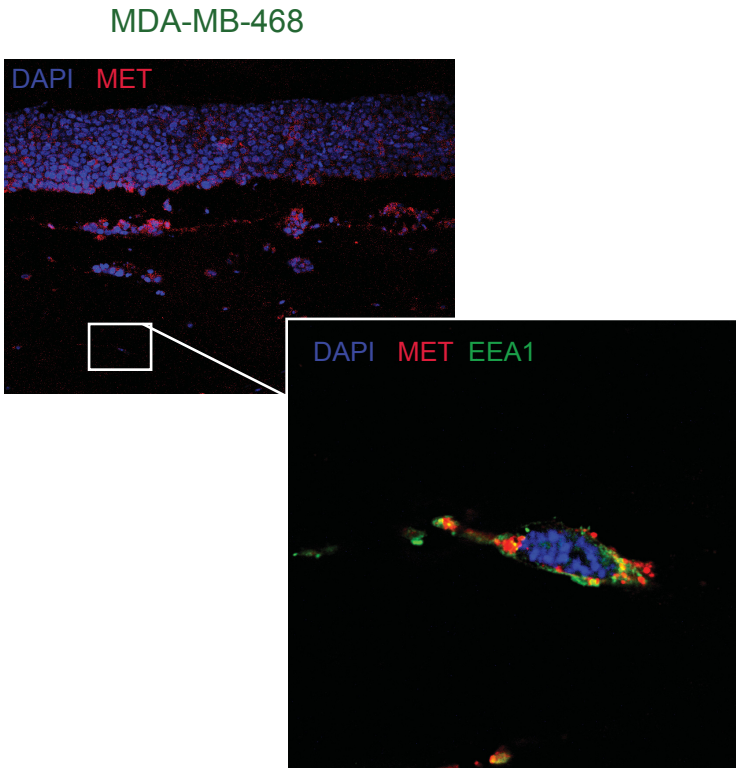


Figure 11: Met is present in endosomes in invading MDA-MB-468 cells
Immunofluorescence on an organotypic section with MDA-MB-468 cells invading into a gel containing MRC-5 fibroblasts. Section was stained with Met (red), EEA1 (green) and DAPI (blue). Image (left) taken using a 20x objective, the insert image (right) of the indicated invading cell was taken using a 63x objective.

8) Aggressive phenotypes correlate with variations in Met trafficking and degradation that suggest an increased endosomal location

The results obtained so far suggested the possible importance for Met to prolong its location on endosomes in aggressive cells. To further investigate this, we extended the study to a wider panel of basal breast cells. Two additional breast cell lines were selected that were also of basal origin[267] and that extended the range of invasive phenotypes studied. These were the MCF-10A cell line, a normal mammary gland cell line that has been immortalised, and the MDA-MB-231 cell line, the most aggressive cell line in the panel, which commonly forms lung, liver and brain mice metastasis following xenografting in immunoincompetent [272].

In a similar fashion to what was observed in the previous cell lines investigated, Met was phosphorylated (Y1349 in the docking site) upon stimulation with recombinant HGF for 15 minutes and, although decreased somewhat, this phosphorylation was sustained over two hours **(Figure 12A)**. Furthermore, the activation of Met was inhibited in the presence of 2μM SU11274, demonstrating the specificity of the activation **(Figure 12A)**.

Treatment with HGF led to the internalisation of Met from the plasma membrane in the two new cell lines and thus was observed in all 4 breast cell lines **(Figure 12B)**. However, while I previously found that the more aggressive MDA-MB-468 cells internalised significantly faster than the MCF10ADCIS.com cells, here I found that the aggressive MDA-MB-231 cells internalised the slowest (25.4%) and the normal MCF10A cells internalised

the fastest (52.4%) after 15 minutes of HGF stimulation **(Figure 12B)**. In fact there were significant differences between the two cell lines at 15, 30 and 60 minutes, showing that, contrary to MCF10ADCIS.com versus MDA-MB-468, the difference is maintained over a long period of time and the MDA-MB-231 cells never manage to “catch up”.

In fact the FACS internalisation assay reveals the Met levels at the plasma membrane over time of HGF stimulation and thus does not reveal a potential recycling of Met. In order to investigate whether Met is recycled upon HGF stimulation in the breast cell lines, I pre-treated the cells with 60μM Primaquine, an inhibitor of recycling from endosomes[273]. Interestingly, there was a tendency for a greater amount of Met to internalise in the aggressive MDA-MB-468 and MDA-MB-231 cells following treatment with Primaquine, while no difference was observed between DMSO or Primaquine treatment for MCF10A and MCF10ADCIS.com cells **(Figure 13A)**. At 15 minutes, 74.5% of Met was present at the plasma membrane in MDA-MB-231 cells, which was reduced to 62.5% following treatment with Primaquine. While Met internalised much quicker in the MDA-MB-468 cells, such that only 51% Met remained at the cell surface upon 15 minutes of HGF stimulation, this however was reduced to 37.6% with Primaquine treatment **(Figure 13A)**. This suggests that in these two aggressive breast cancer cell lines, upon HGF stimulation, a small percentage of internalised Met recycles back to the plasma membrane and possibly re-internalises following restimulation with HGF. The significant difference in Met level at the plasma membrane between the most aggressive cells MDA-MB-231 and the normal cells

MCF10A was lost at 15 minutes following MDA-MB-231 cells treatment with Primaquine **(Figure 13B)**. Therefore, there is a trend for the more aggressive breast cancer cell lines to recycle, perhaps increasing the amount of time spent on endosomes. Thus we hypothesise that in aggressive breast cancer cell lines, a shuttling between the plasma membrane and endosomes may occur for a proportion of Met, resulting in an enhanced endosomal signalling.

These differences in Met internalisation rates and in recycling between the cell lines could possibly be coupled to differences in Met degradation. I therefore checked Met total levels by Western blots in the four cell lines.

Met underwent significant degradation after 2 hours of HGF stimulation in all cell lines ($p<0.01$) except the most aggressive MDA-MB-231 cells **(Figure 14)**. Moreover, the % of Met degradation correlated inversely with the cells' aggressiveness. Thus, it was 24% (but non significant) in MDA-MB-231 cells, 32% in MDA-MB-468 cells, 39% in MCF10ADCIS.com and 49% in MCF10A cells **(Figure 14)**. Thus while the most aggressive MDA-MB-231 cells did not significantly degrade Met at 2 hours, the normal MCF10A cells degraded the most, and the difference between the two cell lines was significant ($p<0.05$) **(Figure 14)**.

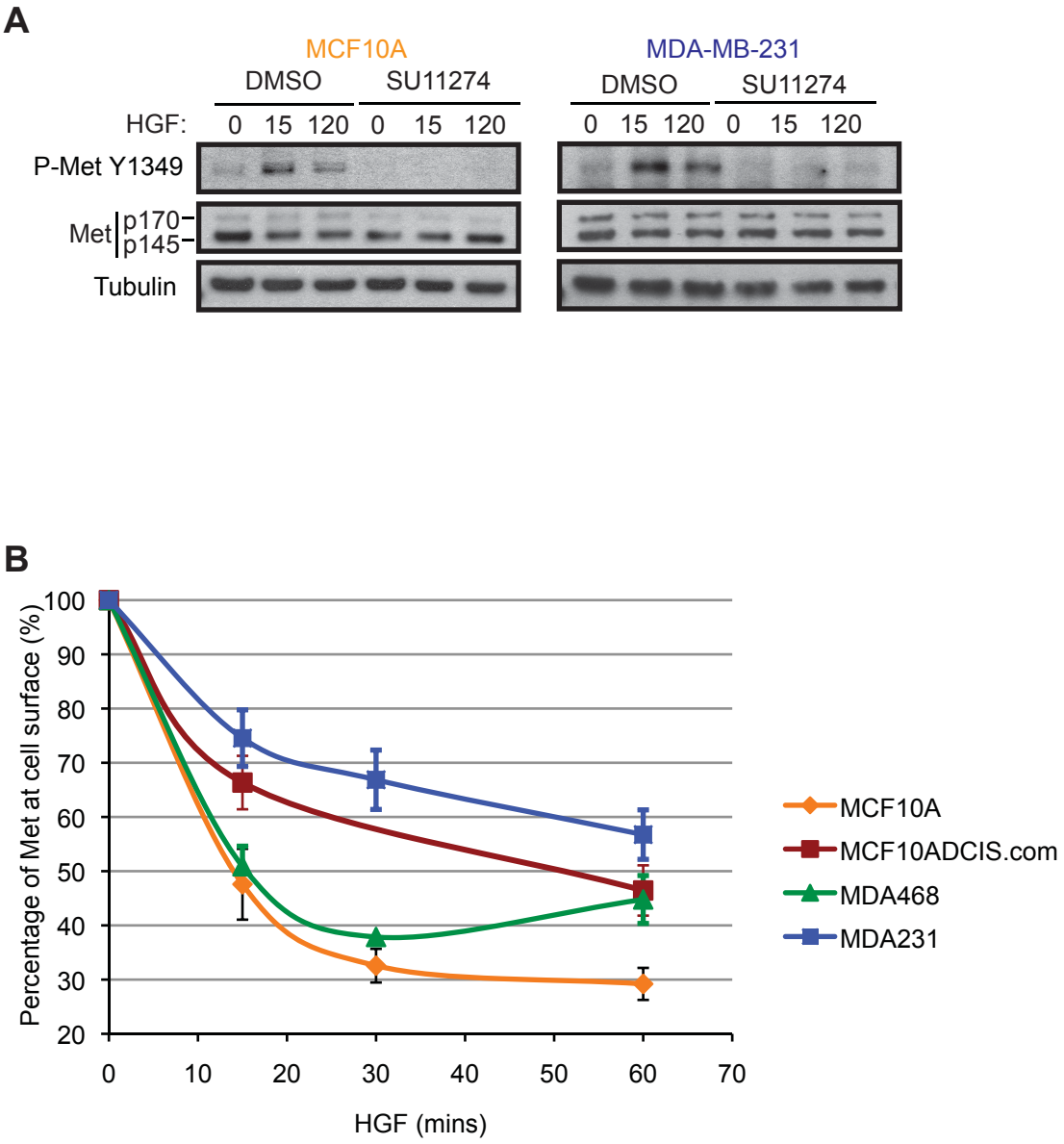


Figure 12: Met is activated upon HGF stimulation in a wide panel of human breast cells

A) Western blots for phospho-Met (Y1349), Met and tubulin upon HGF stimulation (50ng/ml) for 0, 15 and 120 minutes in the presence of DMSO or the Met inhibitor SU11274 (2μM) on lysates from MCF10A and MDA-MB-231 cells. **B)** FACS internalisation assay measuring Met internalisation upon HGF (100ng/ml) in MCF10A, MCF10ADCIS.com, MDA-MB-468 and MDA-MB-231 cells. Data are mean (arbitrary units) ± SEM. (MCF10A (n=4), MCF10ADCIS.com (n=7), MDA-MB-468 (n=9) and MDA-MB-231 (n=5).

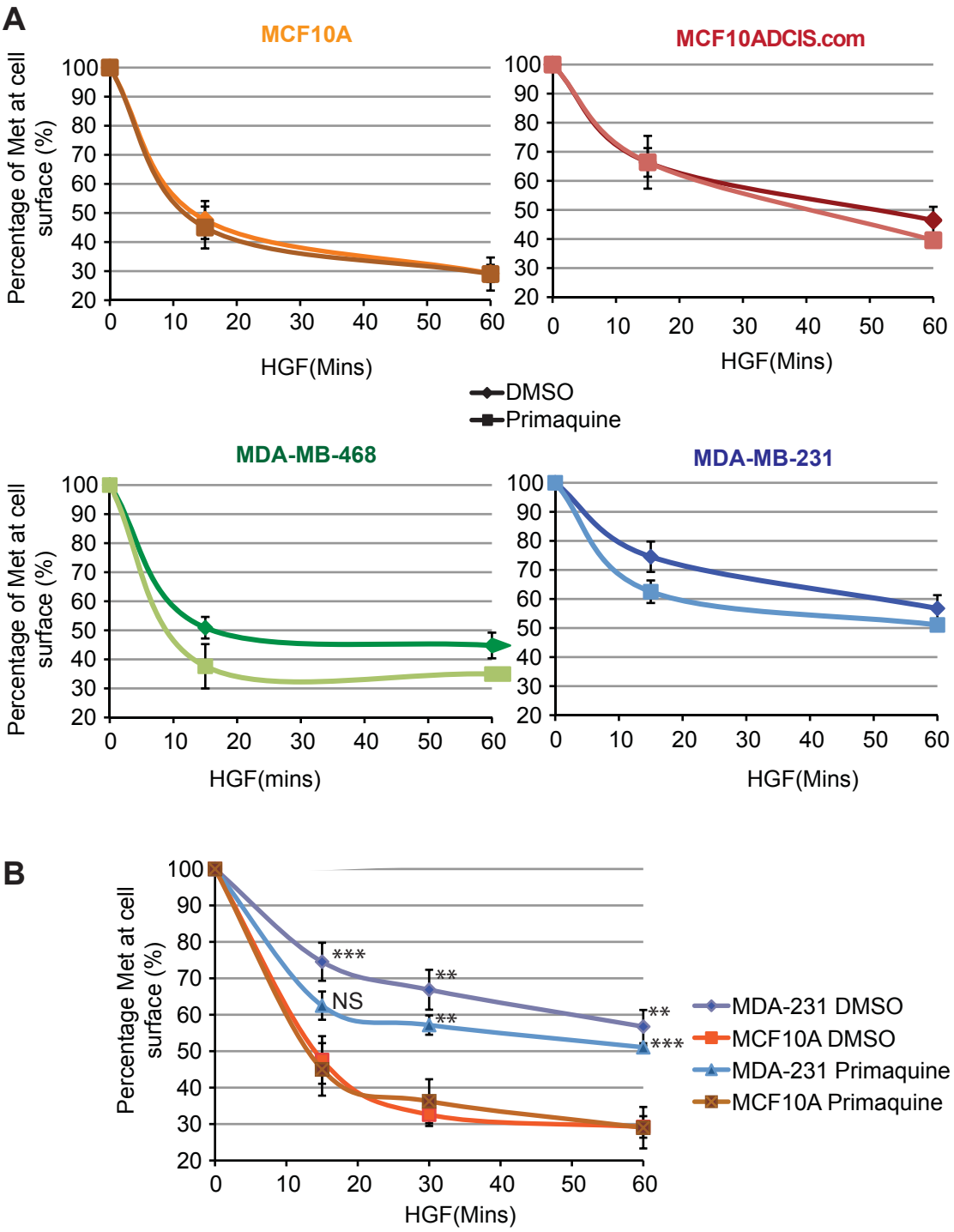


Figure 13: Met tends to recycle more in the more aggressive breast cancer cells

A) FACS internalisation assays measuring Met internalisation upon HGF (50ng/ml) for 0, 15 and 60 minutes in MCF10A (n=3), MCF10ADCIS.com (n=3), MDA-MB-468 (n=4) and MDA-MB-231 (n=4) cells following treatment with DMSO or Primaquine (60μM). **B)** FACS internalisation assays comparing Met internalisation upon HGF(50ng/ml) for 0, 15, 30 and 60 minutes between MCF10A (n=3) and MDA-MB-231 (n=4) cells following treatment with DMSO or Primaquine (60μM). Data are mean (arbitrary units) ± SEM. Significance levels calculated between MDA-MB-231 and MCF10A. *p=<0.05; **p=<0.01; ***p=<0.001; NS=not significant.

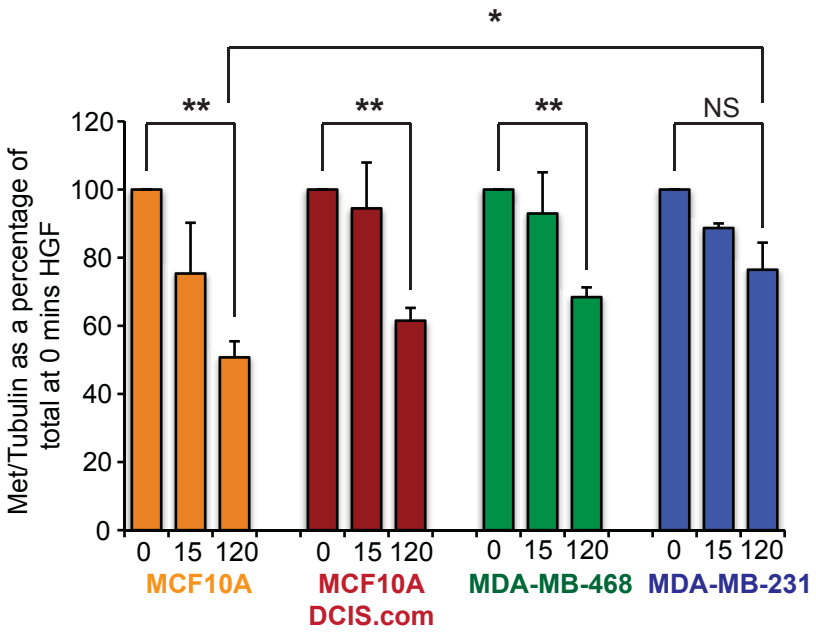


Figure 14: Met is degraded slower in the more aggressive breast cancer cell lines

Quantification of Western blots of Met normalised on tubulin in MCF10A, MCF10ADCIS.com, MDA-MB-468 and MDA-MB-231 cells stimulated with HGF (50ng/ml) for 0, 15 and 120 minutes (n=3). Quantification was performed by densitometry using ImageJ software. Data are mean (arbitrary units) ± SEM. *=p<0.05; **=p<0.01; NS=not significant.

Therefore, there is a difference in Met degradation between the cell lines which correlates with the internalisation rate which occurs and the amount of Met recycling and which, furthermore, correlates with an aggressive phenotype. The normal MCF10A mammary cells exhibit a rapid degradation of Met, which is faster than all the tested cancer cell lines, correlating with its rapid internalisation. Interestingly, the most aggressive breast cancer cell line, MDA-MB-231, has the slowest Met degradation, correlating with its slow internalisation and recycling of Met. These results are summarised in Table 2 and, taken together, they suggest quite strongly that Met may spend more time on endosomes in aggressive versus normal/pre-invasive cells.

We then aimed in investigating the relevance of our findings in breast cancer tissues

	Cell line	Model	Internalisation	Recycling	Degradation	Hypothetic Time spent on endosomes
Aggressiveness - ↓ ++	MCF10A	Normal	++++	-	+++	+
	MCF10ADCIS.com	DCIS	++	-	++	++
	MDA-MB-468	IDC	+++	+	+	+++
	MDA-MB-231	IDC	+	+	-/+	++++

Table 2: Summary of trafficking behaviour of Met in the four breast cell lines.

9) Met expression increases with breast cancer progression

Dr. Colan Ho-Yen looked at the level of Met expression by classical himmunohistochemistry in on human breast cancer samples. Although Met has been shown to be over-expressed in 20-30% of breast cancer[172-174], a comparison between DCIS and IDC has not been reported as far as I can ascertain. We therefore investigated whether Met expression correlated with disease progression. Met expression levels were compared in 47 cases of Ductal Carcinoma In Situ (DCIS) and 153 cases of Invasive Ductal Carcinoma (IDC) containing a mixture of subtypes, including basal-like. It was found that Met expression (both cytoplasmic and plasma membrane) was significantly increased in IDC versus DCIS (**Appendix Figure 1AB and E**), with a greater number of IDC cases having a strong Met staining (17% of DCIS versus 65% of IDC), while more DCIS cases were found to have intermediate (66% of DCIS versus 28% of IDC) or weak (17% of DCIS versus 7% of IDC) Met staining.

10) The levels of Met endosomal localisation in patients' samples correlates with the tumour progression

I next wanted to see if the importance of Met localisation that I have observed *in vitro* on cell lines is also occurring in breast cancer tissues. Therefore, Dr James Hulit, a post-doc in the lab, used immunofluorescence and confocal microscopy analyses to investigate Met endosomal localisation in normal, DCIS and IDC human breast tissue samples. Indeed, Met localisation on endosomes could be visualised on these patients samples **(Appendix Figure 2A**). Although these analyses have so far been performed

on a small number of samples, our results so far are consistent with the ones obtained on the cell lines. Thus a correlation between Met - EEA1 colocalisation and disease progression was found. 25% of Met was present in EEA1 positive compartments in IDC, which was significantly higher than the 15.5% of Met in DCIS, which was also, in turn, significantly higher than the 8.9% in normal breast tissue ($p<0.05$; **Appendix Figure 2B**). Together with the results obtained on the cell lines, our study suggests that there indeed is a correlation between Met endosomal signalling and breast cancer progression to greater malignancy.

DISCUSSION CHAPTER 1

Met endosomal signalling correlates with breast cancer progression

The results presented here on cell lines and breast tissue are consistent with the concept that Met is involved in the promotion and progression of breast cancer to metastasis [174-176]. While both of the cell lines investigated in detail respond to HGF similarly in terms of Met activation and cell migration, there is a correlation between aggressiveness and their ability to invade extracellular matrix in response to HGF.

Moreover, I have shown in this study, that, in breast cell lines, Met gets internalised upon HGF stimulation and signals from endosomes. This endosomal signalling plays a significant role in the activation of Met, ERK1/2 and AKT as well as cell migration in the aggressive MDA-MB-468 breast cancer cell line **(Figure 15)**. I further found that Met presents differences in rates of internalisation, recycling and degradation between four breast lines, of normal, DCIS or invasive phenotype. Our hypothesis is that these differences result in a longer Met localisation on endosomes in aggressive cells versus the normal / DCIS cells. Supporting this, we have demonstrated in human breast cancer tissues, that the endosomal location of activated Met within cancer cells indeed correlates with cancer progression. These results therefore indicate that the trafficking and the endosomal signalling of Met might play a major role in the progression of breast cancer.

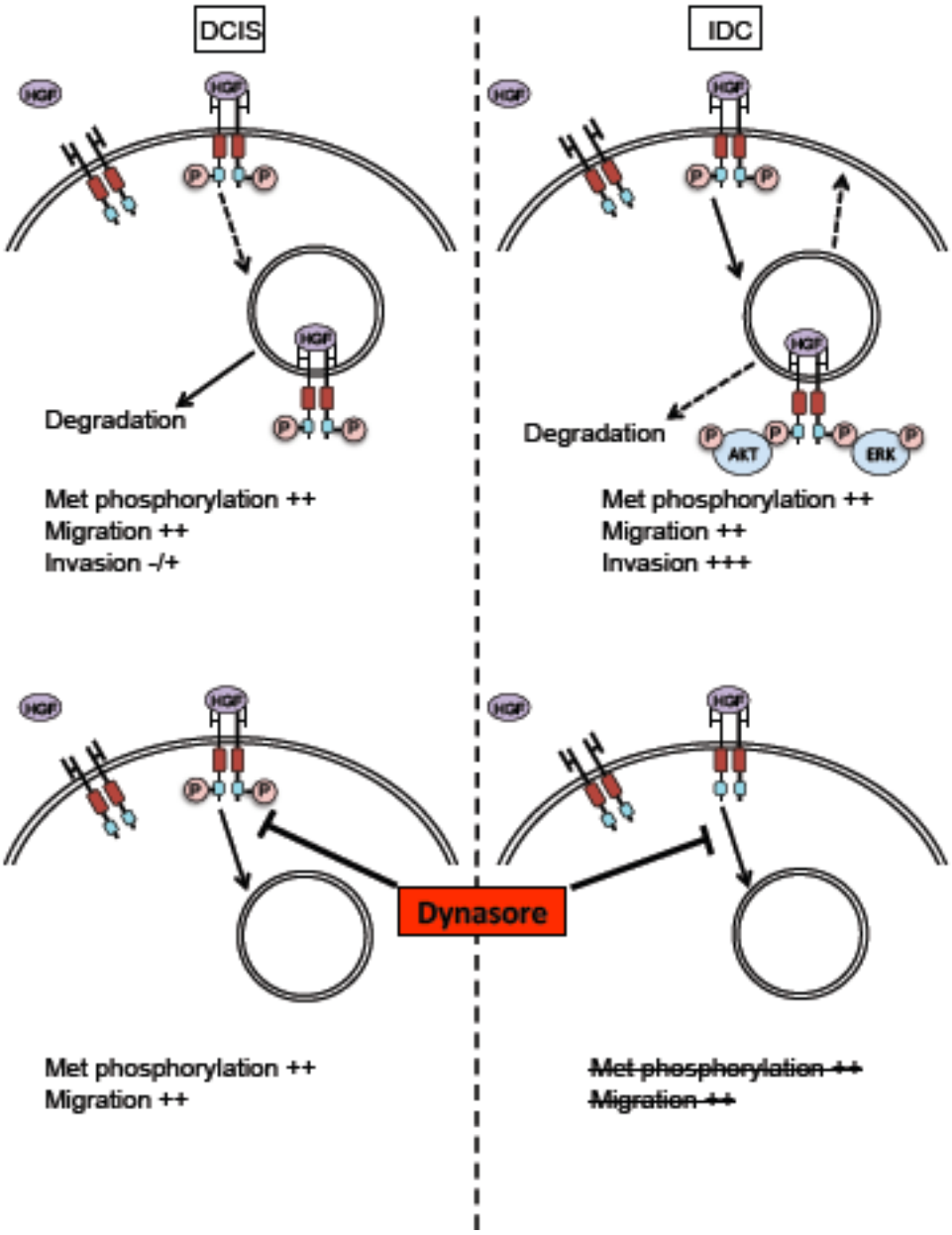


Figure 15: Met endosomal signalling correlates with breast cancer progression

Met can be activated to a similar level and stimulate cell migration similarly in a model of DCIS and IDC. However, Met trafficking is altered in IDC such that Met spends longer on endosomes, where it can activate ERK1/2 and AKT and stimulate cell migration. As a result IDC cells are sensitive to inhibition of endocytosis, using Dynasore, which inhibits Met, ERK1/2 and AKT phosphorylation as well as cell migration.

1) Met expression in breast cancer

Met is expressed in human breast cancer cases

Staining of 47 cases of Ductal Carcinoma In Situ (DCIS) and 153 cases of Invasive Ductal Carcinoma (IDC) revealed that Met expression was significantly higher in IDC compared to DCIS cases. To my knowledge no studies have thus far directly compared Met expression between DCIS and IDC cases. The results obtained in this study strongly suggest that Met may be involved in breast cancer progression, as has been proposed in previous studies through investigating IDC and lymph node metastasis[174-176, 178].

While most studies investigating Met expression in breast cancer have found it to be expressed in 20-30% of cases[172-174], we have found that Met is strongly expressed in 17% of DCIS and 65% of IDC cases, which is much higher than the previously described figures. The set of IDC cases investigated here, contained 17% triple-negative cases, with only 2% basal-like. Thus, the high level of Met expression observed is not due to a higher level of basal breast cancer cases, for which Met has been suggested to be a marker. Nevertheless, it would be interesting to extend this analysis of human breast cancer tissue to look more specifically at whether Met expression is altered in basal-like breast cancer compared to other breast cancer sub-types. Alternatively, it is possible that the set of cases investigated here had an unusually low patient survival, due to the fact that Met expression has been associated with a poor prognosis[175]. Therefore it would be interesting to obtain the patient survival data for the cases we have investigated in this study and compare them to Met expression levels.

Furthermore, we have taken into account both membrane and cytoplasmic Met staining in this analysis, while many other studies may have solely investigated plasma membrane staining (or when both plasma membrane and cytoplasmic staining were present together[176]), perhaps believing that cytosplasmic staining was in some way unspecific.

Met is expressed in a panel of breast cancer cell lines that represent the basal breast cancer phenotype

The normal MCF10A as well as the three breast cancer cell lines used throughout this study have basal-like gene expression[267], they are ER/PR/Her2 negative[267] and express EGFR[268, 274].

Each of the cell lines express endogenous Met, in accordance with previous studies on these cell lines[180, 274-276] as well as studies showing Met is a possible marker for basal breast cancer[175, 182, 277].

This use of these basal-like cells makes this study therapeutically relevant since patients with basal breast cancer currently have a poor prognosis due to both the aggressiveness of the disease and to a lack of effective treatment resulting from a lack of markers to target.

2) Met signalling and its influence on the behaviour of breast cancer cells

Met is specifically activated to comparable levels upon HGF stimulation in the pre-invasive MCF-10ADCIS.com cells and the more aggressive MDA-MB-468 cells.

We have shown that HGF stimulates Met phosphorylation in each of the four cell lines. I have investigated the Y1349 Met docking site and, while this is the site that is most representative of the activity of Met functionally, it also would be interesting to look at the Y1234/5 site to investigate the intrinsic kinase activity of Met.

Met phosphorylation was calculated in the MCF10ADCIS and MDA-MB-468 lines and interestingly, cells from both have a similar level of Met phosphorylation (around 6 fold) following stimulation with HGF. The two major downstream signalling pathways, ERK 1/2 and AKT, were then investigated. We detected that HGF significantly activates ERK1/2 and AKT in MDA-MB-468 cells (**Figure 3**) despite the fact there is a basal activation of AKT, possibly resulting from the lack of PTEN expression[278] in these cells.

In MCF10ADCIS.com cells, we could not detect the activation of ERK1/2 and AKT following HGF stimulation, probably due to the fact they already were highly activated under basal conditions, despite serum starvation (**Figure 1**). This may be due to the expression of the h-Ras oncogene[265]. Since HGF stimulates these cells' wound healing and migration, the activation of a more extensive range of signalling molecules could be looked at by Western blot analysis, for instance JNK, FAK, PLC-γ and SHC.

HGF stimulates the migration of MCF10ADCIS.com cells and of MDA-MB-468 cells to a similar level but affects their invasion differentially.

HGF increased the healing of wounded monolayers of both the MCF-10ADCIS.com and MDA-MB-468 cells to a similar degree. However, since wound-healing assays are measuring a result of a mixture of cell function, including cell migration and proliferation, I also performed Transwell migration assays, which offer a more accurate study of migration alone as they were performed over a much shorter time frame (4 hours versus overnight). In this assay, both of the cell lines responded to HGF by increasing their migration. Perhaps surprisingly, the more aggressive MDA-MB-468 cells had the same level of migration upon HGF stimulation as the MCF-10ADCIS.com cells. However, this was also not completely unexpected as both MCF10ADCIS.com cells and MDA-MB-468 cells have similar levels of Met expression and activation upon HGF stimulation.

Nevertheless, a difference between the two cell lines was observed in their ability to invade in response to HGF secreting MRC-5 fibroblasts embedded in an extracellular matrix of an organotypic 3D model. The MCF-10ADCIS.com cell line only showed a small amount of invasion. This is not surprising as it is a model for DCIS, which is the pre-invasive stage of breast cancer. Nevertheless, HGF recently has been shown to induce invasion of MCF-10ADCIS.com cells in a 2D co-culture system with fibroblasts[180]. By contrast, the MDA-MB-468 cells, which represent an aggressive stage of breast cancer, showed substantial invasive activity, which was largely due to Met activity as SU11274 significantly and strongly reduced the invasion.

Obviously MRC5 may secrete growth factors or cytokines other than HGF and despite the fact that optimisation experiments showed that EGFR, VEGFR and FGFR were not involved in the increased Transwell migration towards MRC-5 cells, these receptors may still be involved in the invasion of these cells. To fully establish this, it would be necessary to check the phosphorylation status of a variety of RTKs by Western blot. Nevertheless, again, the strong reduction of cell invasion in the presence of the Met inhibitor allows us to use this model to follow Met-dependent invasion.

In summary, these studies indicate that while there is a similar level of Met activation and cell migration upon HGF stimulation in both the MCF10ADCIS.com and MDA-MB-468 cells, the MDA-MB-468 cells have a significantly higher level of HGF-induced invasion.

3) <u>Endosomal signalling of Met</u>

Met internalisation is dependent on dynamin and can be impaired to a similar level in MCF-10ADCIS.com cells and in MDA-MB-468 cells

I have shown that HGF stimulates HGF bound Met internalisation in the four cell lines studied. Previously, Met has been shown to be internalised by clathrin and dynamin dependent mechanisms[63, 82]. Pre-treatment of MCF10ADCIS.com and MDA-MB-468 cells with dynasore, a pharmacological inhibitor of the GTPase dynamin, prevented internalisation of approximately 95% of Met in both cell lines. Thus, this method was used to compare the effect of endocytosis inhibition on Met signalling and stimulation of cell migration in both cell lines.

Because a pharmacological compound may trigger side effects, it would be interesting to confirm these results comparing the influence of a dominant negative dynamin mutant versus a wild type dynamin construct[159].

Due to the fact that dynamin is involved in the endocytosis of many cargos through controlling clathrin-dependent and -independent internalisation mechanisms[279], blocking dynamin likely leads to much broader inhibition than just Met. However, since in my studies I have always compared the effect of dynamin inhibition on HGF stimulated events (as compared to no stimulation) and since I could verify that Met endocytosis was efficiently impaired, it is reasonable to think that the results observed are the consequence of Met endocytosis inhibition. It would be interesting however to inhibit Met endocytosis with other methods, if possible more specific approaches than the pharmacological inhibition used here, in order to confirm these results.

I attempted to knock down clathrin heavy chain (CHC) by RNAi using Amaxa technology. However, I was not able to achieve an appropriate level of CHC diminution in these breast cancer cells for a functional effect to be observed. One possibility would be to impair the function of specific regulator(s) of Met endocytosis but so far, very little is known regarding which regulators are likely to be the significant ones. A few clathrin adaptors have been reported to control Met endocytosis. For example, overexpression of AP180 could be used[82]. Knocking down the adaptor and ubiquitin ligase c-Cbl could also be used, as I have successfully shown this to inhibit Met endocytosis (see

Chapter II). Given more time, this would improve the specificity over dynamin inhibition since c-Cbl specifically controls the endocytosis of RTKs[65].

Met remains activated post-endocytosis in breast cancer cells

In the two breast cancer cell lines investigated in detail (MCF10ADCIS.com and MDA-MB-468), Met was found to be recruited in early endosomes, where it colocalised with a phospho-tyrosine staining. These results are consistent with previous studies in HeLa cells[82] and suggest that Met is activated on endosomes in both breast cancer cell lines. Similar results have been obtained with a phospho-Met antibody, however the quality of the staining is not very good. This is the reason I used a general phospho-tyrosine antibody (clone 4G10). To obtain a more specific result, I could use the Proximity Ligation Assay (PLA), which allows the detection of two associated proteins by immunofluorescence, with a high level of sensitivity and specificity. This technique utilises specially designed secondary antibodies with a probe, containing a small fragment of DNA, attached. Closely associated probes ligate and following an amplification step, highly visible colocalisations between two desired molecules can be observed by fluorescence microscopy[280]. This could be used to more specifically observe the location of phosphorylated Met with a higher degree of sensitivity using Met and phospho-tyrosine or Met and phospho-Met antibodies.

The aggressive MDA-MB-468 cells require endocytosis for full Met activation and signalling

In MCF10ADCIS.com and MDA-MB-468 cells, where Met is activated following HGF stimulation to the same fold increase (around 6, see **Figure 1**) Blocking endocytosis strongly reduced this phosphorylation in MDA-MB-468 cells, while there was no decrease in Met phosphorylation in the MCF10ADCIS.com cells. These results suggest that, although in both cells Met appears to be activated on endosomes (**Figure 4**), the less aggressive cell line has a lower requirement for this spatial restriction for the activation of Met. This is quite a remarkable finding, as to our knowledge, a requirement for endocytosis for the phosphorylation of an RTK itself upon stimulation with its ligand has not been reported. It would be very interesting to see if endocytosis is also required for the phosphorylation of the Y1234/5 site in the kinase domain of Met, or whether it is only required for the Y1349 site in the MDA-MB-468 cells.

The comparison could not be made between the 2 cell lines regarding ERK1/2 and AKT as they were not stimulated by HGF in the MCF-10ADCIS.com cells. In order to fully examine the requirement for endocytosis for Met signalling in the MCF-10ADCIS.com cells, it would be necessary to investigate more signalling pathways. Meanwhile, In MDA-MB-468 cells, levels of Met, ERK1/2 and AKT phosphorylation were reduced significantly upon endocytosis inhibition, indicating that these cells require endocytosis to fully activate Met and downstream signalling pathways.

These results suggest that, while the endocytosis of Met may not be very important for its activation of Met in the less aggressive MCF-10ADCIS.com cells, endosomal signalling plays a large role in the activation of downstream signalling pathways in the more aggressive MDA-MB-468 cell line.

Signalling from the endosome versus from the plasma membrane

Vieira et al. showed that while ERK1/2 and PI3K signalling is hypophosphorylated in endocytosis defective cells upon EGF stimulation, PLC-γ and SHC are hyperphosphorylated, suggesting that these molecules do not require EGFR endosomal signalling for their full activation[145]. It would therefore be interesting to investigate the signalling of both PLC-γ and SHC in our cell models to see if there is also a subset of molecules that signal from the plasma membrane, while other molecules signal from endosomes. Perhaps, the MCF-10ADCIS.com cells have a larger requirement for PLC-γ and SHC signalling. ERK1/2 activation through the endosomal signalling of the EGFR and Insulin receptors has been previously demonstrated[145, 147], while studies on HeLa cells have shown that the endosomal signalling of Met leads to ERK1/2 activation[62]. However, in contrast to EGF activated cells not relying on endocytosis for activation of SHC, activation of SHC by the insulin receptor does depend on intact endocytosis machinery[147]. This suggests that different RTKs may activate molecules at different localisations within cells. It would be interesting to investigate in the breast cancer cell lines whether there are any molecules activated by Met whose signalling does not take place from endosomes as previously shown for the EGF receptor[145].

4) <u>Met endosomal signalling and influence on breast cancer cell migration</u>

Interestingly, my results have demonstrated that upon endocytosis inhibition, while the HGF dependent migration was not modified significantly in the MCF-10ADCIS.com cells, it was significantly reduced by almost 60% in the more aggressive MDA-MB-468 cells (**Figure 7**). These results are consistent with the ones obtained on Met activation and signalling and demonstrate that the endosomal signalling of Met is required for the migration of the aggressive MDA-MB-468 breast cancer cells. The fact that Dynasore did not affect the basal cell migration suggests that at least over the time-frame of the experiment, Dynasore does not lead to non-specific effects or toxicity.

A few studies have investigated the role of RTK endocytosis in cell migration. Jékely et al. demonstrated that RTK endocytosis is important for directional cell migration towards ligands during *Drosophila* development[149]. This finding was further developed by Assaker et al., who found that fully functional trafficking between the plasma membrane and endosomes, involving both Rab5 and Rab11, was required to keep RTKs active at the leading edge of migrating border cells and consequently for directional cell migration[150].

Importantly, a few studies have demonstrated a requirement for endocytosis in cell migration upon Met activation. Palamidessi et al. showed in HeLa cells that Rac activation upon HGF requires clathrin- and rab5-dependent endocytosis, which is consequently required for mesenchymal cell migration[158]. Work from our lab, in which I was involved, has showed that prevention of the internalisation and accumulation on endosomes of a

constitutively active Met receptor, reduces Rac activation, cell migration and experimental metastasis [159] (see Chapter II-2). However, so far, no studies have demonstrated a requirement for RTK endocytosis in breast cancer cell migration.

Interestingly, in this study I have observed Met on endosomes in invading MDA-MB-468 cells in a 3D organotypic invasion assay, suggesting that the endosomal signalling of Met may also provide an advantage for invading cancer cells. In addition, the 3D organotypic cultures used could provide a valuable preclinical model to test endocytic inhibitors as a potential future cancer therapy and it would be very interesting to test Dynasore on a panel of breast cancer cell lines in this assay.

Studies by Wang et al. have shown that the endosomal signalling of EGFR is sufficient to promote cell proliferation and survival in BT20 breast cancer cells[144]. Cancer cells often have increased survival, compared to non-transformed cells, providing them with the ability to survive in the bloodstream and then at the site of metastasis. Therefore, both proliferation assays and further survival assays could be performed on the cell lines with and without Dynasore, in order to assess the role of Met endosomal signalling in the survival of breast cancer cells.

5) Correlation between Met endosomal signalling and breast cancer progression

Differences in RTK trafficking and degradation in four breast cell lines.

Interestingly, I observed differences in trafficking in the four breast cell lines studied, which are summarised in **Figure 16**. Although it is not possible to make definite conclusions on only four cell lines, especially as the differences are not “clear cut”, nevertheless our results *suggest* that the consequences of the observed differences in Met trafficking/degradation in the four cell lines result in Met being more accumulated on endosomes in aggressive versus DCIS and even more versus normal cells.

In the normal mammary cells, MCF10A, Met is internalised rapidly but was also rapidly degraded following HGF stimulation, suggesting that, in such cells, Met does not remain long on endosomes.

In the MCF10ADCIS.com cells, Met is internalised more slowly upon HGF stimulation but degrades rather quickly, although slower than in the normal MCF10A cells. In addition, Met activation and Met dependent cell migration were not affected by Dynasore treatment, suggesting that these cells do not depend on endosomal location for Met signalling, perhaps because the majority of Met signalling takes place from the plasma membrane. All together, these results suggest that, in MCF10A.DCIS cells, Met does not stay too long on endosomes.

In the aggressive MDA-MB-468 cells, Met has a significantly faster internalisation upon HGF addition than in the MCF10ADCIS.com cells, but it

degrades more slowly and, moreover, a proportion of internalised Met recycles and probably undergoes cycles of internalisation / recycling (as long as the ligand is present). In addition, Met activation and Met dependent cell migration were impaired by Dynasore treatment. These results suggest that Met remains for a longer period of time on endosomes in these cells.

Finally, in the aggressive MDA-MB-231 cells, upon HGF stimulation, Met internalises and degrades the slowest. Additionally a small percentage of Met seems to recycle in these cells. I have not yet obtained any data on the requirement for endocytosis for Met signalling and Met dependent cell functions in these cells, but it will be very interesting to see if Met endosomal signalling is important in these cells.

Thus, again, although the differences between the cells are not all obvious, our results suggest that Met trafficking is modified with the aggressiveness of the breast cancer cells. The differences are quite clear if looking at opposite ends of the spectrum. Thus the normal MCF10A cells have the fastest internalisation and subsequent degradation of Met upon HGF stimulation, while the aggressive MDA-MB-231 cells have the slowest internalisation and the slowest degradation of Met, which also appears to undergo recycling upon HGF stimulation. Additionally, we find that only the two cell lines representative of IDC seem to have some Met recycling occurring upon HGF stimulation and that the level of Met degradation decreases with the cells' aggressiveness.

We therefore hypothesise that through modification in its internalisation and/or recycling characteristics, together with modification of its degradation kinetics, Met accumulation on endosomes increases with the progression of breast cancer. We could imagine that cancer cells modify Met trafficking in order to maintain it longer on endosomes, from where it could sustain transforming signals.

This work is ongoing however and, in order to verify our hypothesis, I am planning to quantify Met endosomal localisation in the four cell lines following HGF stimulation through analysing confocal pictures. As a control, the transferrin receptor will be investigated using fluorescently labelled transferrin. This would indicate if the differences we observe are specific to Met or are due to a general difference in receptor trafficking between the cell lines, which could, for example, occur as a result of some overexpression of Rab proteins involved in vesicle trafficking. I also will analyse the influence of Dynasore on Met activation, signalling and Met dependent migration in MCF10A and MDA-MB-231 cells. Furthermore, in the future this work will be extended to investigate a larger panel of breast cancer cell lines.

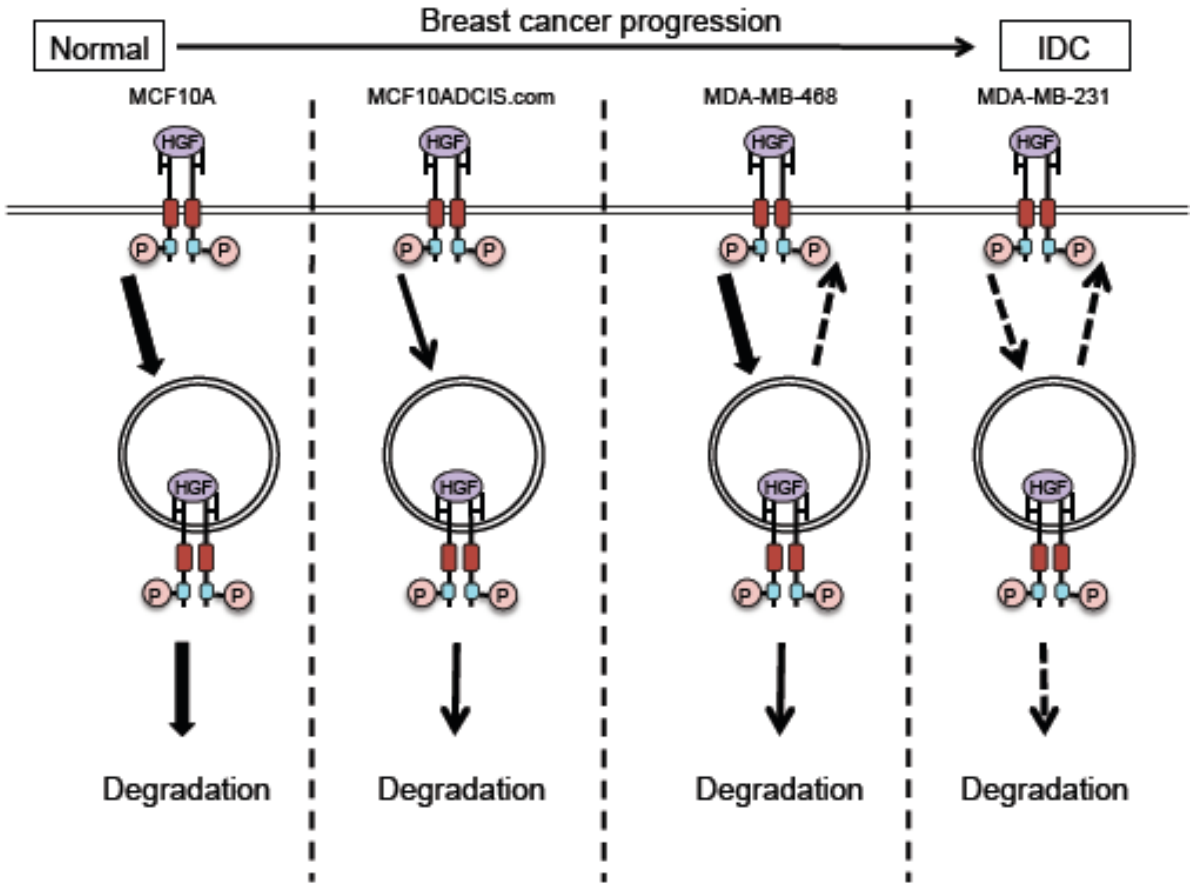


Figure 16: Changes in Met trafficking during breast cancer progression
In normal mammary cells; MCF10A, Met is internalised and degraded rapidly following HGF stimulation, suggesting that Met does not stay long on endosomes. In the MCF10ADCIS.com cells, Met is internalised more slowly but degrades rather quickly, although slower than the normal MCF10A cells, suggesting that Met has an increased life-span but does not stay too long on endosomes. Finally, there are the models of IDC: the MDA-MB-468 cells, where Met has a significantly faster internalisation but a slower rate of degradation compared to MCF10ADCIS.com cells; the MDA-MB-231 cells, in which Met internalises the slowest but also degrades the slowest. Moreover a proportion of internalised Met recycles in both of these cell types. Altogether these data are suggesting that Met spends a longer time on endosomes in these aggressive cells.

In the experiments investigating recycling, I have only observed a tendency for Met to recycle in the MDA-MB-468 and MDA-MB-231 cells using the drug primaquine. I am planning to use other tools to impair recycling such as a Rab11 dominant negative construct as we did in NIH3T3 cells expressing mutant Met[159]. Given more time, I could perform Biotinylation Recycling Assays, which would provide a clear indication of any Met recycling that is occurring in these cells. It is also worth noting, that the investigation of Met degradation in the breast cancer cell lines was not performed in the presence of cycloheximide. Thus, it may be possible that over a two hour time period, new Met has been synthesised and processed by the cells and any future experiments, particularly over longer periods of time, should be performed in the presence of cycloheximide to prevent protein synthesis.

Correlation between Met endosomal localisation and breast cancer progression on tissue samples

A few studies have investigated Met localisation in breast cancer using immunohistochemistry, with conflicting results; one correlating cytoplasmic staining with a significantly shortened survival[172], and the other correlating plasma membrane staining with tumour aggressiveness[176].

Our observation by immunofuorescence and confocal microscopy of Met on endosomes in human breast tissue was very exciting; as this has not been reported as far as I am aware.

Moreover, we observed an increase in the amount of Met present on endosomes in breast cancer as compared to normal breast tissue, with the greatest amount observed in invasive breast cancer. This supports my observations on the cell lines and is consistent with our hypothesis that an

endosomal localisation of Met correlates and thus may be involved in tumour progression. We need however to extend this study on a larger number of cases to increase its clinical relevance.

"Addiction" to endosomal signalling

The idea that cancer cells alter Met trafficking in order to increase the amount of time Met spends on endosomes suggests that cancer cells are "addicted" to "endosomal signalling". The concept of "oncogene addiction" has been around for many years. Originally described by Weinstein in 2002[281], "oncogene addiction" illustrates the high dependence of cancer cells on one particular oncogene and how suppression of this oncogene is detrimental for cancer cells[282]. This forms the basis of many types of novel cancer therapy approaches today. However, perhaps it is also possible for cancer cells to be "addicted" to endosomal signalling, due to the multitude of possible advantages this location may provide to a signalling RTK.

A few studies have demonstrated a deregulation in trafficking of RTKs in breast cancer cell models, leading to decreased degradation and/or increased recycling of RTKs. The EGFRvIII mutant, found in many cancers including breast cancer[191, 192], has a defect in ubiquitination, internalisation and degradation of the receptor, as it is unable to undergo correct phosphorylation of its c-Cbl binding site[193].

In fact, of particular interest to the cancer research community are the studies by Caswell et al.[252] and Muller et al.[262] on EGFR and Met recycling

respectively. They demonstrated that H1299 cells (non-small cell lung cancer cells) containing mutant p53 have increased recycling of EGFR or Met together with $\alpha5\beta1$ integrin in an RCP dependent manner[255, 262]. In Chapter 3, I investigate the role of $\beta1$ integrin in Met signalling, the results obtained here suggest that this may also be worth investigating in breast cancer cells. Interestingly, the MDA-MB-231 and MDA-MB-468 cells used in my study both have a mutant p53[283], which may explain why I may have observed a small amount of Met recycling. It would therefore be interesting to investigate the potential role of RCP in Met recycling and signalling in MDA-MB-231 and MDA-MB-468 cells.

Interestingly, RCP has been associated with breast cancer progression, with its overexpression leading to normal MCF10A cells acquiring tumourigenic properties with increased activation of Ras and ERK1/2[187].

Other endocytic regulators have also been found to be deregulated in breast cancer. For example, Rab11a has been shown to be overexpressed in DCIS and induce proliferation and increased MAPK signalling in MCF10A cells upon EGF stimulation, through recycling of EGFR[194]. Whilst an increased expression of Rab25 in breast cancer patients is an indicator of poor prognosis[190].

RCP, Rab11a and Rab25 are all involved in vesicle recycling and therefore it is not clear whether their overexpression would play a part in tumourigenesis through a decrease in RTK degradation or the increased presence of activated RTKs on endosomes. Most of these studies demonstrate persistent signalling due to a prolonged lifespan of activated receptors through decreased degradation or increased recycling. So far, to my knowledge,

nothing has been shown demonstrating a difference in RTK internalisation and trafficking towards endosomes or a role for *endosomal signalling* in breast cancer progression.

Thus, our study, performed both on cell lines and in human breast cancer tissues, suggest a correlation between breast cancer progression and the amount of time spent on endosomes by Met. Further investigation on a wider panel of breast cancer cell lines as well as a larger set of breast cancer tissue samples, will reveal whether this is indeed the case.

RESULTS CHAPTER II

Understanding Met oncogenic Mutants' signalling and targeting this in tumourigenesis.

Under normal conditions, Wt Met is activated by its ligand HGF, after which it is internalised and traffics to early endosomes. Met continues to signal from early endosomal compartments, and in fact, this location is required for Met signalling to ERK 1/2[62]. However, Met usually is degraded efficiently, thereby putting a stop to continuous Met signalling.

Recent work from our lab has investigated the trafficking of the D1246N and M1268T constitutively active Met mutants that have previously been identified in papillary renal cell carcinoma[202]. Carine Joffre showed that M1268T Met mutants have increased internalisation and decreased degradation compared to Wt Met[159]. The mutants constitutively internalise and traffic to endosomal compartments from where they signal, and then recycle back to the plasma membrane. It is proposed from these studies that the accumulation of the M1268T and D1246N mutant Met on endosomes leads to a persistent endosomal signalling, cell transformation and *in vivo* tumourigenesis.

The aim of my work was to understand mechanisms regulating the increased endocytosis on the one hand (Part 2) and the decreased degradation on the other hand (Part 3), of Met mutants, and impair these mechanisms in the view of reducing their oncogenicity. I describe my contribution to the recent

publication (Joffre, Barrow et al, NCB 2011) and I present my original unpublished work. Initially, I have studied the sensitivity of both mutants to three Met TKIs, presented in part 1.

1) Sensitivity of Met mutants D1246N and M1268T to Met specific inhibitors *in vitro* and *in vivo*.

a) Met mutants are highly and constitutively phosphorylated.

It has previously been shown that the three Met mutants are highly constitutively activated as compared to Wt Met [284]. I performed Western blot analyses from NIH3T3 cells expressing murine Met Wt, D1246N or M1268T, which confirmed that both the D1246N and M1268T Met mutants are highly activated in both the kinase domain (Y1234/5) (**Figure 1A**) and the docking site (Y1349) (data not shown). This is to a much higher degree than in the Wt cells, either non-stimulated or stimulated with 5, 50 or 200ng/ml HGF for 5, 10 or 20 minutes (**Figure 1B**).

In the clinic, the response to RTK inhibitors of patients bearing cancers expressing RTK oncogenic mutations can be highly modified toward increased sensitivity or resistance. Therefore, I aimed to investigate the sensitivity of these two mutants to small molecule Met inhibitors.

b) M1268T mutant Met is sensitive to Met inhibitors while the D1246N mutant is resistant.

I tested various different pharmacological small molecule inhibitors of Met: PHA-665752 (PHA), SU11274 (SU) and PF-2341066 (PF) (which is orally available) on WT, M1268T and D1246N Met expressing cells.

All three inhibitors were found to greatly reduce the phosphorylation of the M1268T mutant in a dose dependent manner (**Figure 2ABC**). Met phosphorylation was completely inhibited in these cells with PF at 0.025μM, PHA at 0.1μM and SU at 1μM **(Figure 2ABC)**.

The D1246N mutant, however, was found to be resistant to the three inhibitors, although it did appear to be slightly sensitive to higher doses of PF, though these differences were not significant (**Figure 2ABC**). To investigate this further, I tested higher doses of PF on the D1246N cells. At the very high dose of 5μM, D1246N Met phosphorylation (Y1234/5) was completely inhibited (**Figure 3A**). At a dose of 1μM, phosphorylation of D1246N Met was reduced by 62%, while an investigation into lower doses of PF on M1268T cells revealed that M1268T phosphorylation was reduced by 65% at 0.01μM. Therefore the M1268T mutant is 100 fold more sensitive to PF than the D1246N mutant. Moreover, it is likely that the drug has lost its specificity towards Met at doses higher than 1μM[225] and it can be concluded that the D1246N mutant is resistant or, at least, poorly sensitive to PF. It is worth noting here that all three inhibitors also reduced the phosphorylation of the Wt Met treated with HGF as shown for treatment with PF **(Figure 3B)**.

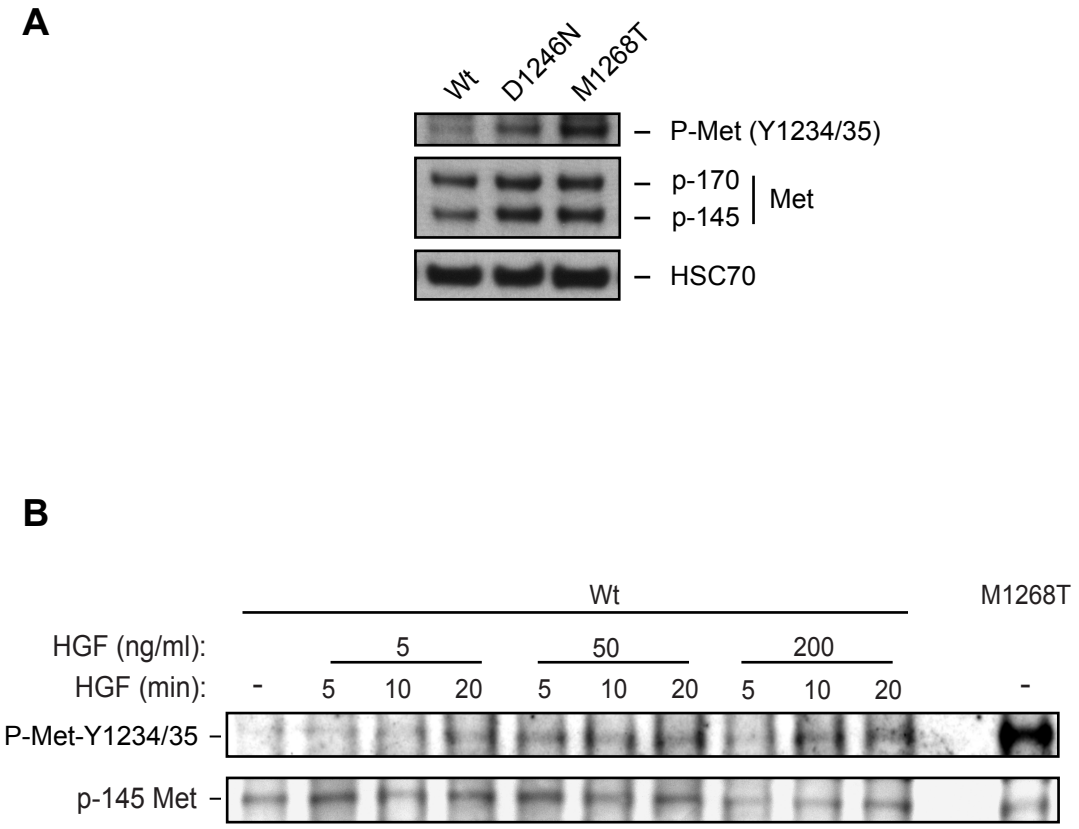


Figure 1: M1268T mutant is phosphorylated to a higher degree than Wt cells + HGF

A) Western blot for phospho-Met (Y1234/35), Met and HSC70 on Wt, D1246N and M1268T cell lysates. **B)** Western blot for phospho-Met (Y1234/5) and Met on Wt cells stimulated with either 5, 50 or 200 ng/ml HGF for 5, 10 or 20 minutes and the unstimulated M1268T cells.

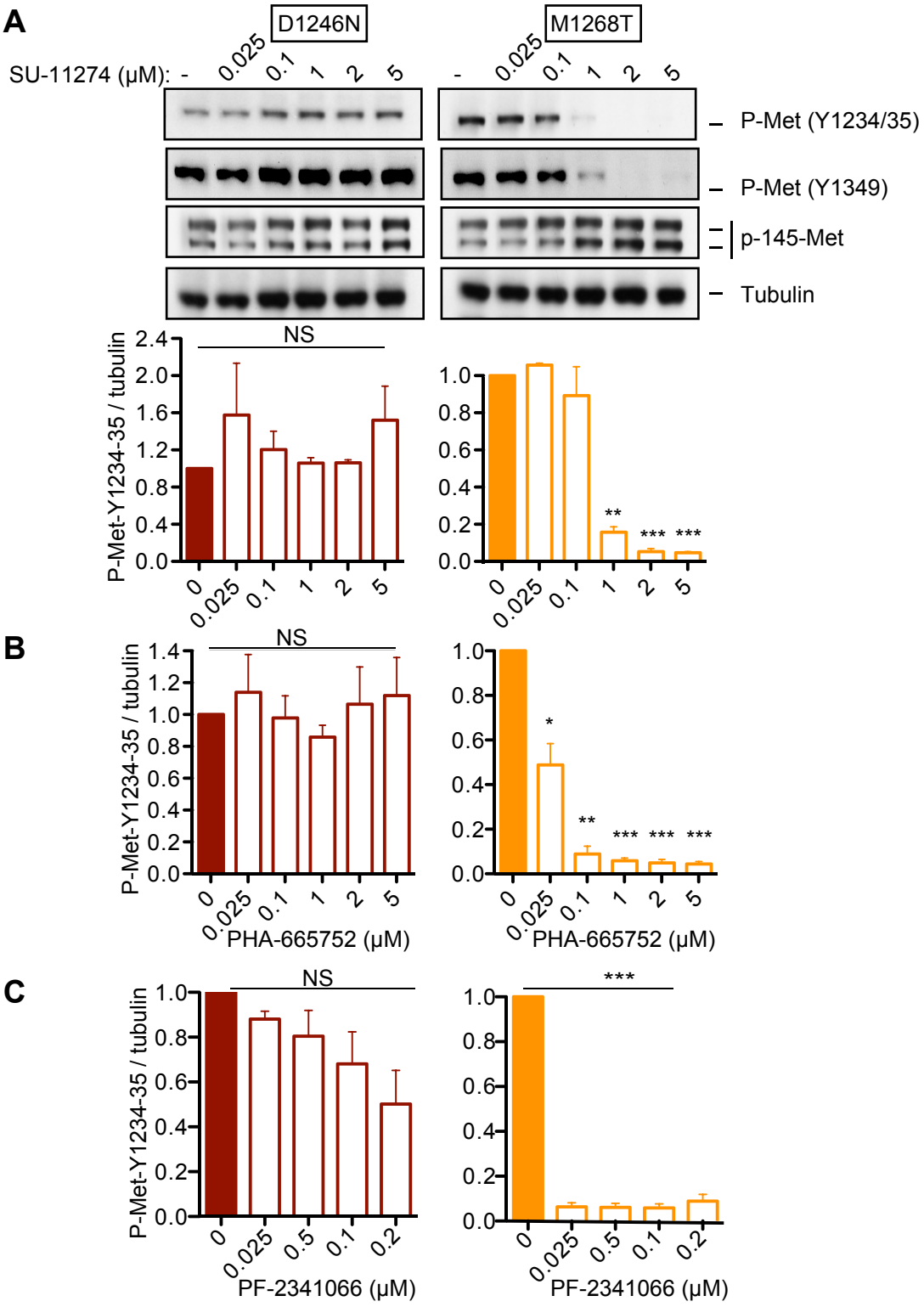


Figure 2: M1268T cells are sensitive to various small molecule Met inhibitors, while the D1246N cells are resistant

A) Western blots for phospho-Met (Y1234/35 and Y1349), Met and tubulin in D1246N and M1268T cells treated with stated doses of SU-11274. Bottom panel is quantification of the blots of D1246N and M1268T cells.

BC) Quantification of Western blots of D1246N and M1268T cell lysates treated with stated doses of **B)** PHA-665752 and **C)** PF-2341066. Data are mean (arbitrary units) ± SEM. **=p<0.01; ***=p<0.001; NS=not significant.

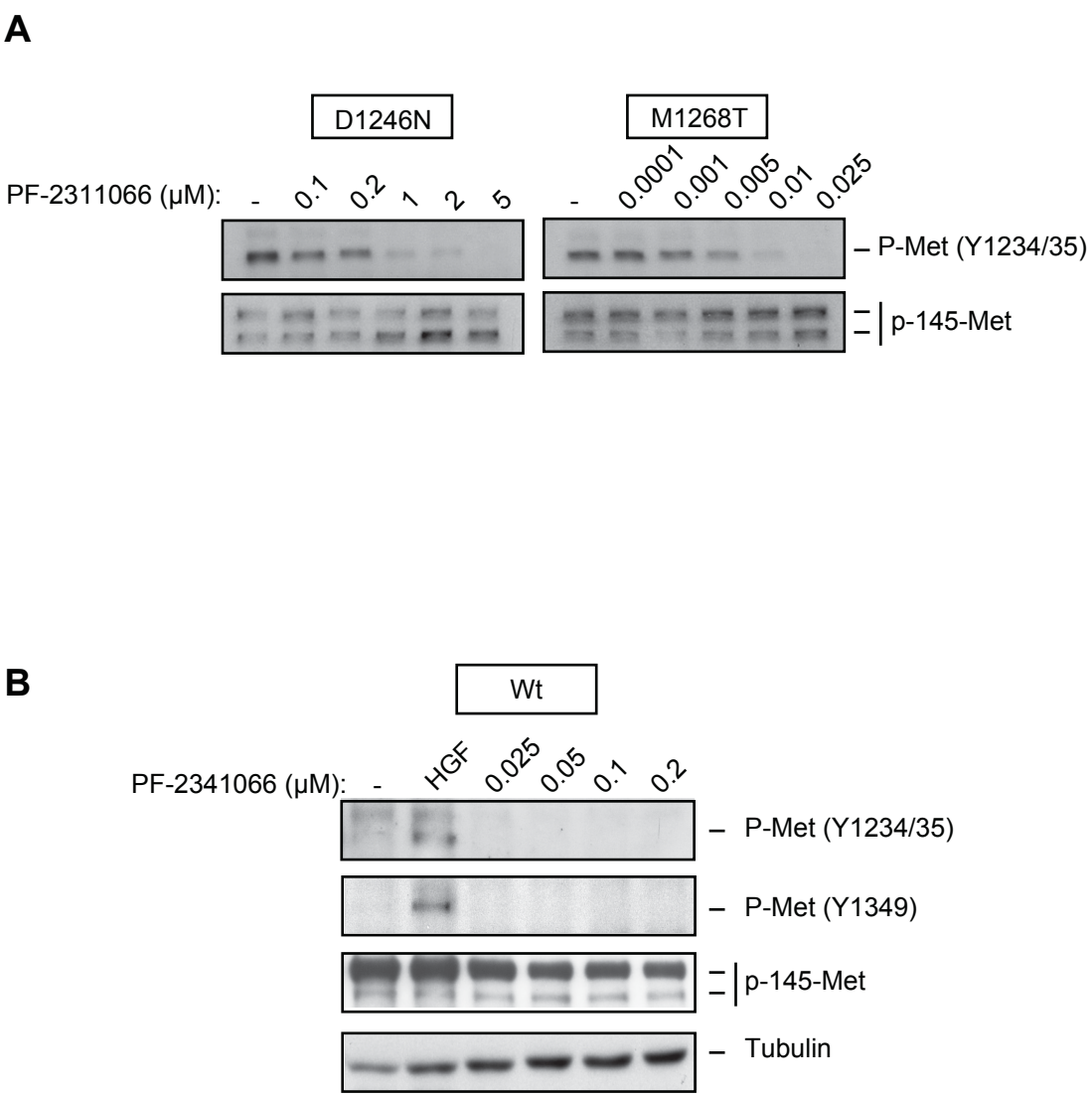


Figure 3: M1268T cells are 100 fold more sensitive to PF-2311066 than the D1246N cells.
A) Western blot for phospho-Met (Y1234/35) in D1246N and M1268T cells treated with low or high dose of PF-2341066 respectively (n=1). **B)** Western blot for phospho-Met (Y1234/35 and Y1349), Met and tubulin in Wt cells stimulated with HGF or not (-) and treated with the stated doses of PF-2341066.

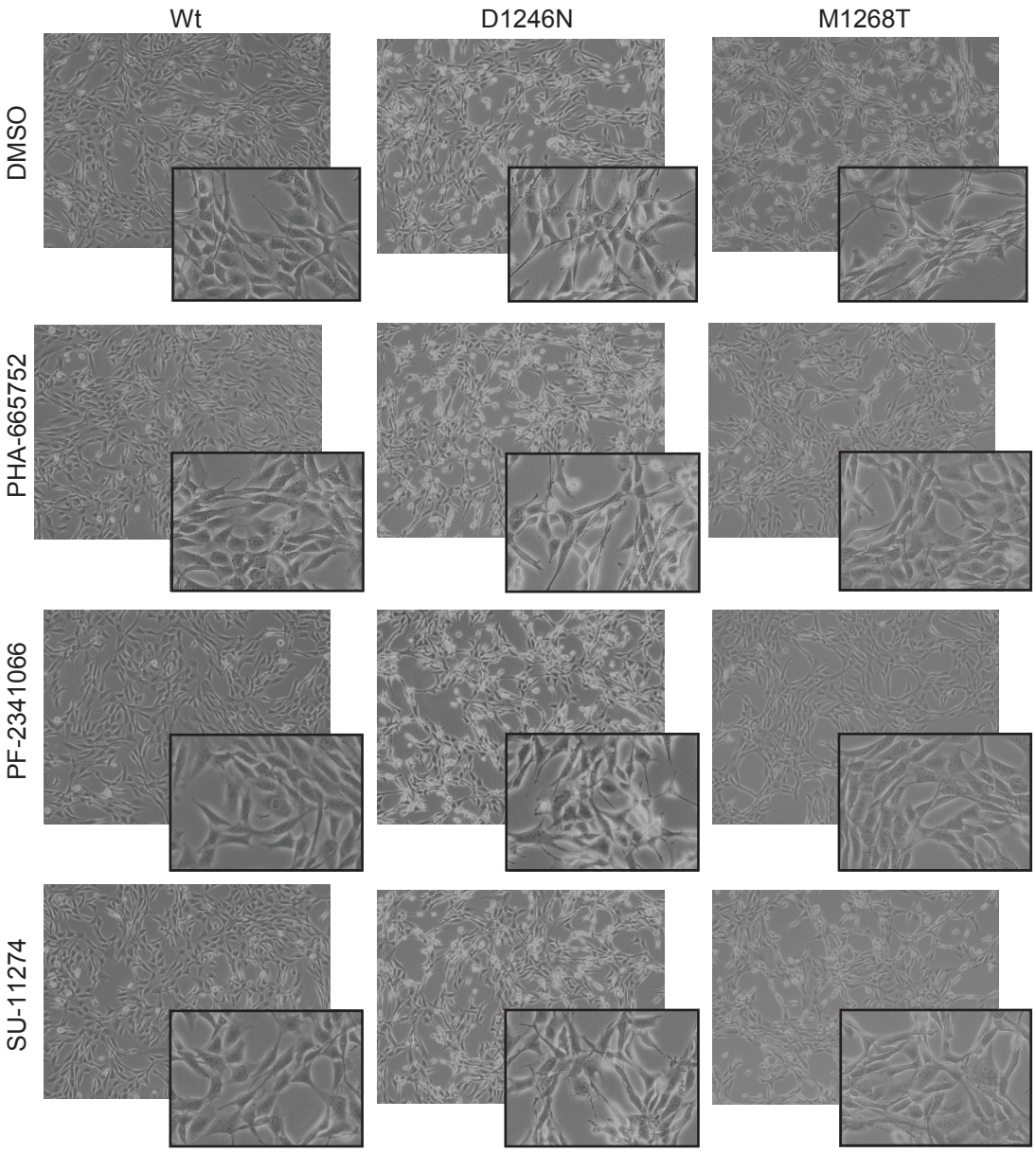


Figure 4: Met inhibitors alter the morphology of the M1268T cells but not of the D1246N cells

Wt, D1246N and M1268T cells were treated with either DMSO, PHA-665752 (0.1μM), PF-2341066 (0.2μM) or SU-11274 (2μM) overnight, after which pictures were taken and morphological changes observed.

The M1268T and D1246N mutant expressing cells have a distinct morphology, being more rounded and loosely adherent as compared to the Wt cells (**Figure 4**). The effect of treatment of the M1268T cells with the various inhibitors over 24 hours could be seen in a change in cell morphology, such that they came to resemble more closely the Wt morphology with a flattened and more spread appearance, while no effect on the morphology of the D1246N cells was observed. No change was observed in Wt cells either, consistent with the fact that the poorly phosphorylated Wt Met requires HGF to reach a threshold of phosphorylation necessary for cell transformation (**Figure 4**). I then investigated further the potential differences in the actin cytoskeleton organisation by staining the cells with phalloidin-555. While only around 30% of Wt cells lacked stress fibres, around 70% of D1246N and M1268T cells lacked stress fibres **(Figure 5AB)**. Treatment with PHA significantly reduced the number of M1268T cells lacking stress fibres but had no effect on the D1246N cells, while silencing of Met with Met RNAi **(Figure 5C)** reduced the number of both D1246N and M1268T cells lacking stress fibres **(Figure 5AB)**. Furthermore, treatment with either PHA-665752 or transfection with Met RNAi had no effect on the stress fibres of the Wt expressing cells **(Figure 5AB)**.

These results indicate that Wt Met is not transforming, that the lack of stress fibres observed in D1246N and M1268T cells is due to the expression of the Met mutants, and that the D1246N cells are resistant to the small molecule Met inhibitors used in this study.

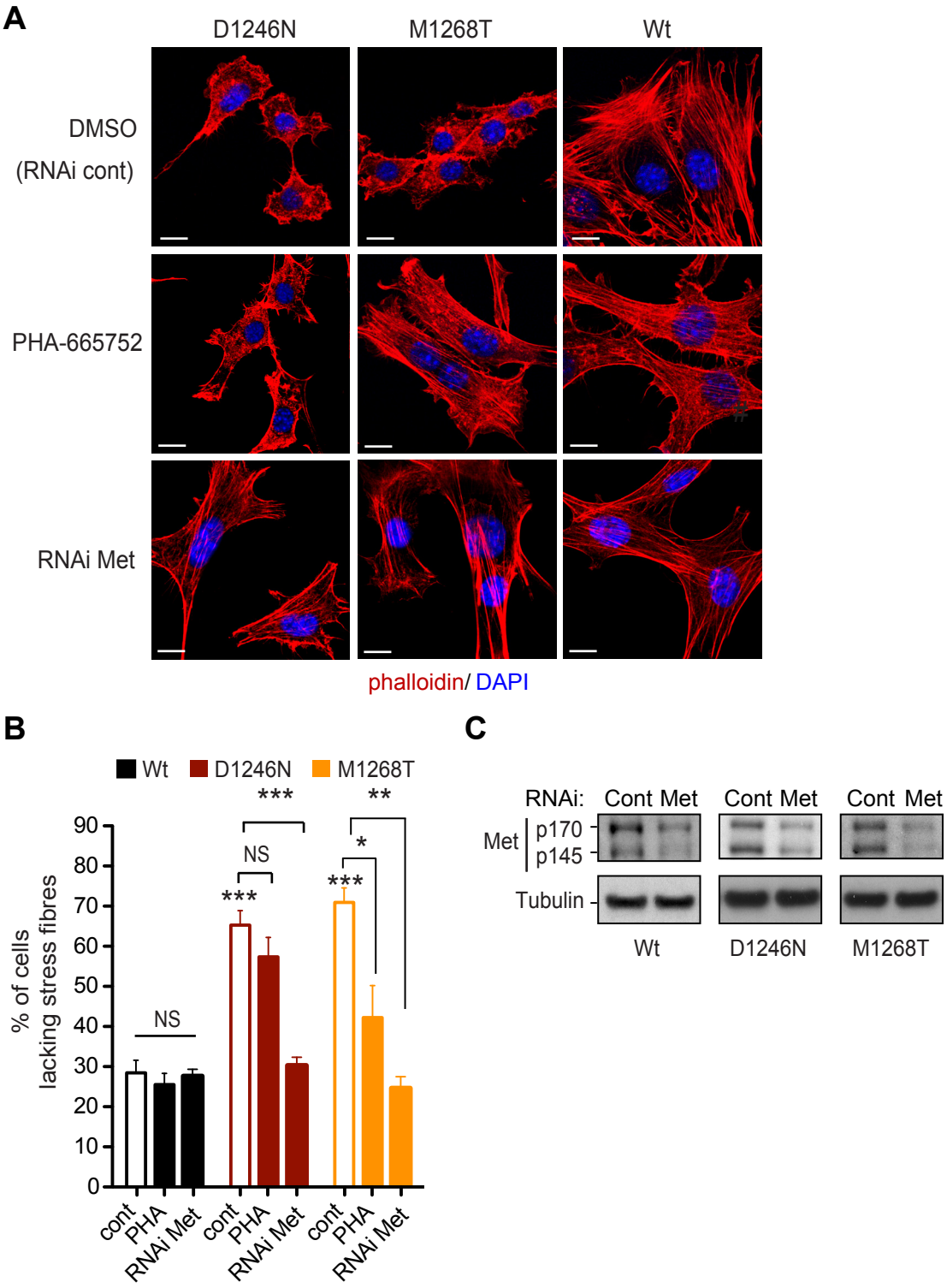


Figure 5: Treatment with Met inhibitors restores stress fibres of M1268T but not D1246N cells

A) Confocal sections of Wt, D1246N, M1268T cells stained with Cy3-phalloidin (red) and DAPI (blue), following treatment with DMSO or PHA-665752 for 90 minutes or transfected with control or Met RNAi. Scale bars: 10 µm. **B)** Graph shows the mean percentage of cells lacking stress fibres ± SEM (n=3). Cont is the average of DMSO (n=3) and RNAi control (n=3). **C)** Western blots for Met and tubulin expression. *p<0.05; **p<0.01; ***p<0.001; NS=not significant.

The M1268T and D1246N mutants have previously been shown to be more tumourigenic both *in vitro* and *in vivo* [203, 285]. I performed several biological assays to assess the influence of the Met inhibitors on cellular migration, growth in anchorage independent conditions and *in vivo* tumorigenesis.

In Transwell migration assays, the M1268T mutant expressing cells migrate 7-fold more than the Wt cells ($p<0.05$) **(Figure 6A)**. The D1246N mutant cells are less migratory than the M1268T mutant cells but both of these Met mutant expressing cells still migrate more than the Wt cells stimulated with HGF in Transwell migration assays (**Figure 6A**). Moreover, HGF can increase further the migration of the D1246N and M1268T cells[159].

Treatment with the Met inhibitors PHA and PF significantly reduced the migration of the M1268T cells by over 50% ($p<0.05$), however the D1246N cells remained resistant to the Met inhibitors with no decrease in cell migration (**Figure 6B**). Nevertheless both mutant cells have a significantly reduced migration following Met knockdown (**Figure 6C**), while, neither treatment with Met inhibitors nor transfection with Met RNAi had an effect on the migration of Wt Met expressing cells.

These results show that the increased migratory ability of the cells expressing Met mutants is dependent on the Met mutations and that the cells expressing Met mutant D1246N are insensitive to Met inhibitors fwith regard to affecting their increased cell migration.

The growth in soft agar of the Met mutant cells was also investigated, as this measures the capability of cells to undertake anchorage independent growth; a prerequisite for cancer development and a necessary prelude to

metastasis. Colonies were allowed to form in soft agar after which they were treated daily with fresh media for 4 days. As compared to Wt cells, the M1268T and D1246N mutant expressing cells formed a significantly larger number of colonies ($p<0.01$) (**Figure 7A**). Following treatment with media containing DMSO or PF (0.1μM), PHA (0.1μM) or SU (2μM) for the last 4 days the number of M1268T colonies was greatly reduced as well as the colony area (data not shown) by at least 50% (**Figure 7BC**), reaching a similar number and colony area (data not shown) to that formed with Wt cells (**Figure 7C**). Furthermore, none of the inhibitors had an effect on the number of colonies or colony area (data not shown) formed by the WT or D1246N cells (**Figure 7BC**). Meanwhile, transfection with Met RNAi significantly reduced the number of colonies grown in soft agar of both D1246N and M1268T cells, while there was no effect on Wt cells (**Figure 7D**).

Both the soft agar and Transwell migration assays demonstrate that the D1246N cells are resistant to the Met inhibitors in terms of their biological functions, which is consistent with the results obtained on D1246N phosphorylation.

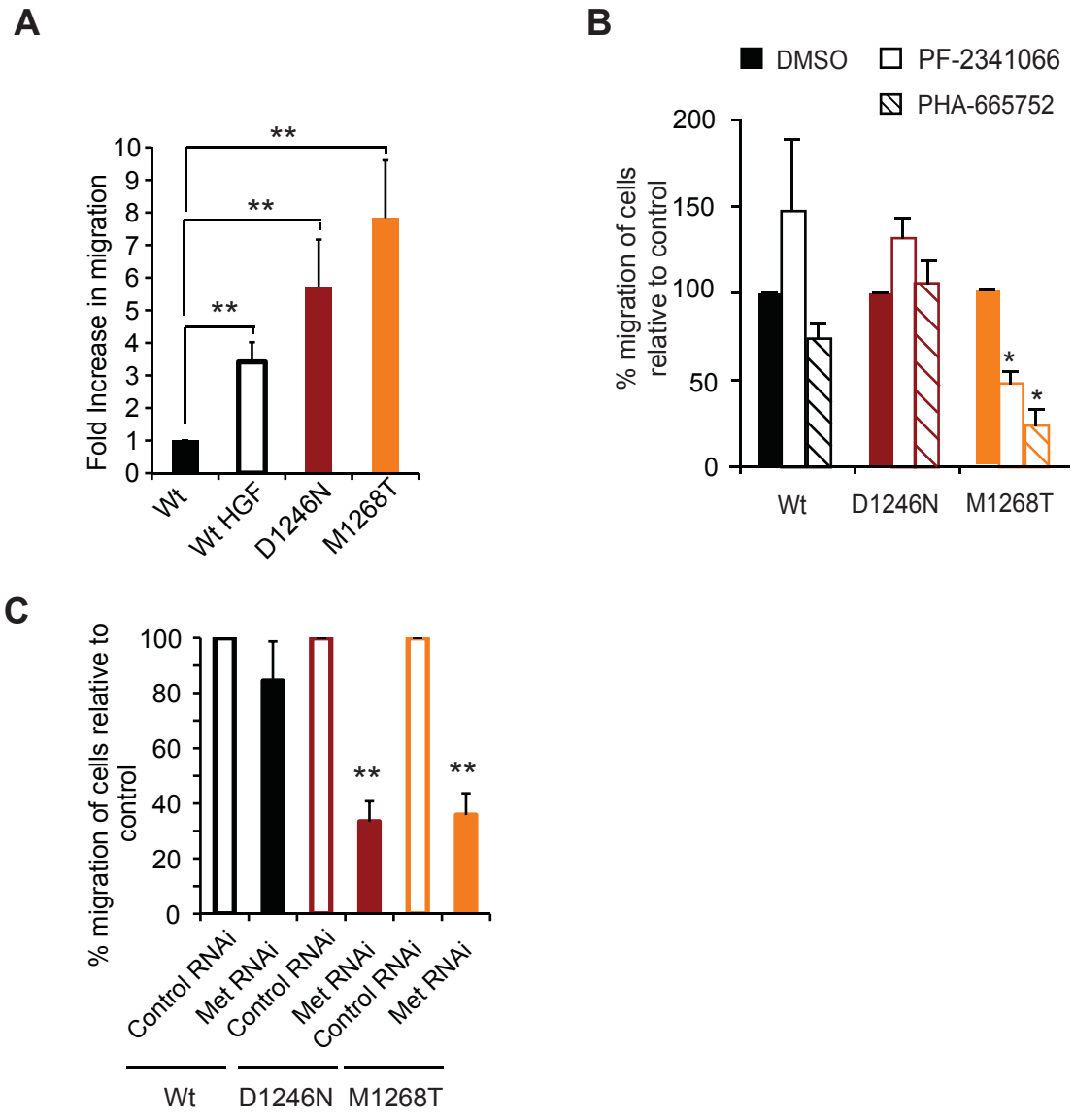


Figure 6: Treatment with Met inhibitors reduces cell migration of M1268T, but not of D1246N, cells

ABC) Transwell migration assays monitoring Wt, D1246N and M1268T cells migration towards a chemoattractant (complete media). A) Without or with HGF (50ng/ml). After 2 hours the number of cells migrated through Transwell pores were counted. B) Cells were treated with DMSO, PF-2341066 (0.2μM) or PHA-665752 (0.1μM). C) Cells were transfected with Control or Met RNAi (n=3). Data are mean (arbitrary units) ± SEM. *p<0.05; **p<0.01.

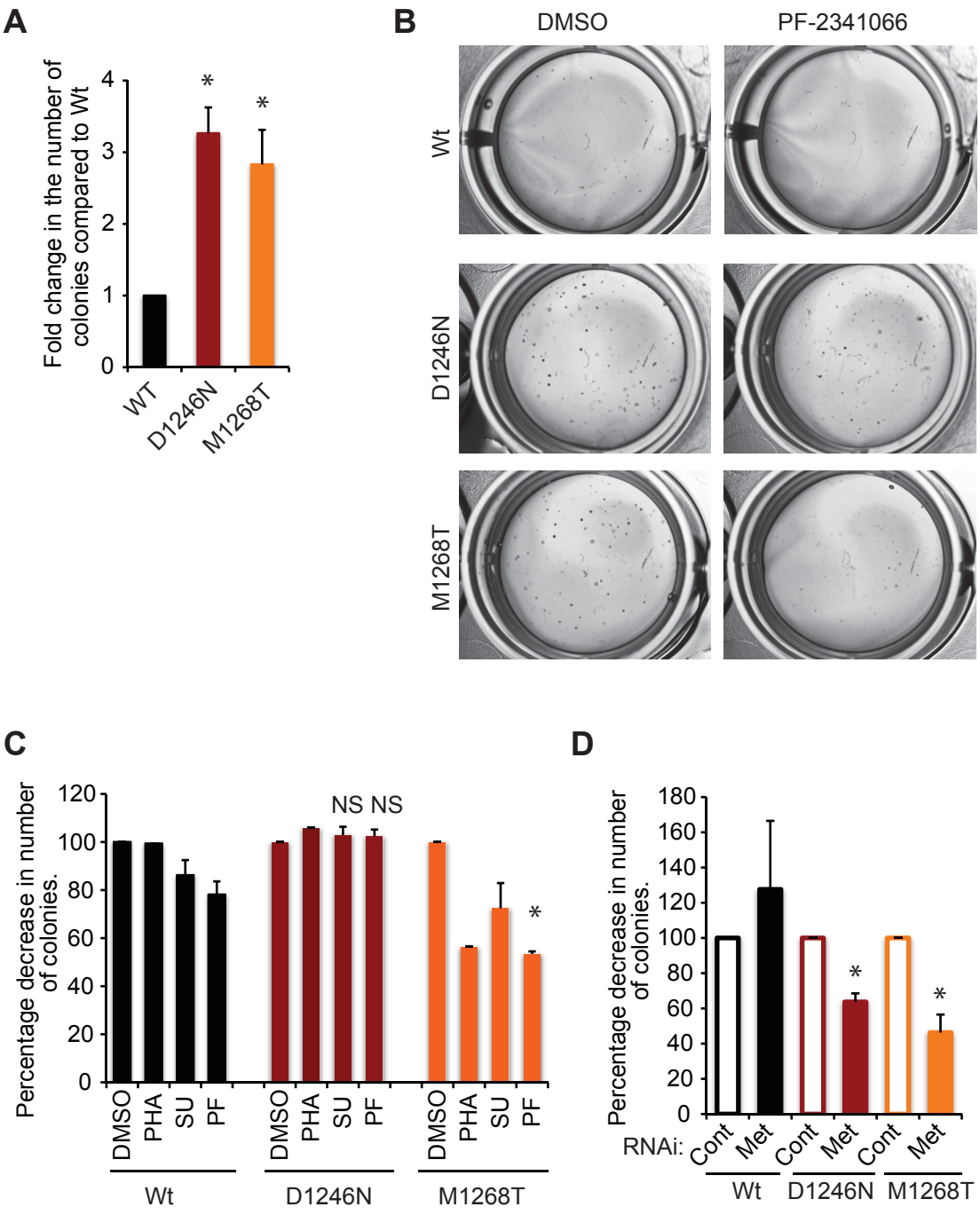


Figure 7: Treatment with Met inhibitors prevents anchorage independent growth of M1268T but not D1246N cells

A-D) Wt, D1246N and M1268T cells were cultured in soft agar. Experiments were performed in triplicate. **A)** The graph shows the number of colonies compared to Wt cells. **B)** From day 5, DMSO or PF-2341066 (0.2μM) was added daily to the medium. Pictures taken at day 9. **C)** The graph shows the percentage decrease in the average number of colonies ± SEM following treatment with PHA-665752 (0.1μM) (n=1), SU-11274 (2μM) (n=2 for M1268T, n=3 for D1246N) or PF-2341066 (0.2μM) (n=3) compared to DMSO control. Each condition is in duplicate. **D)** The graph shows the percentage decrease in the number of colonies following transfection with Control or Met RNAi (n=3). Data are mean (arbitrary units) ± SEM.*p<0.05; NS=not significant.

In vivo, Wt, D1246N and M1268T mutant cells form tumours in nude mice when grafted subcutaneously. The M1268T and D1246N mutant cells form tumours very quickly and are palpable within 3 days, while the Wt cells are slower to form tumours (data not shown). Once tumours reached 50mm^3, I applied either DMSO or PF in DMSO topically to the skin over the surface of each tumour. The M1268T grafted cells underwent a significant decrease in tumour growth while the D1246N cells did not respond to the treatment (**Figure 8**). Due to the fact that PF is orally available, Carine Joffre also treated mice with subcutaneous Wt, D1246N, M1268T tumours by oral gavage and a similar decrease in tumour growth was observed in M1268T tumours, while there was no effect on Wt or D1246N tumours (data not shown). This confirms that the *in vitro* results we have obtained where the M1268T cells are sensitive and the D1246N cells are resistant to Met inhibitors also applies to *in vivo* tumourigenesis.

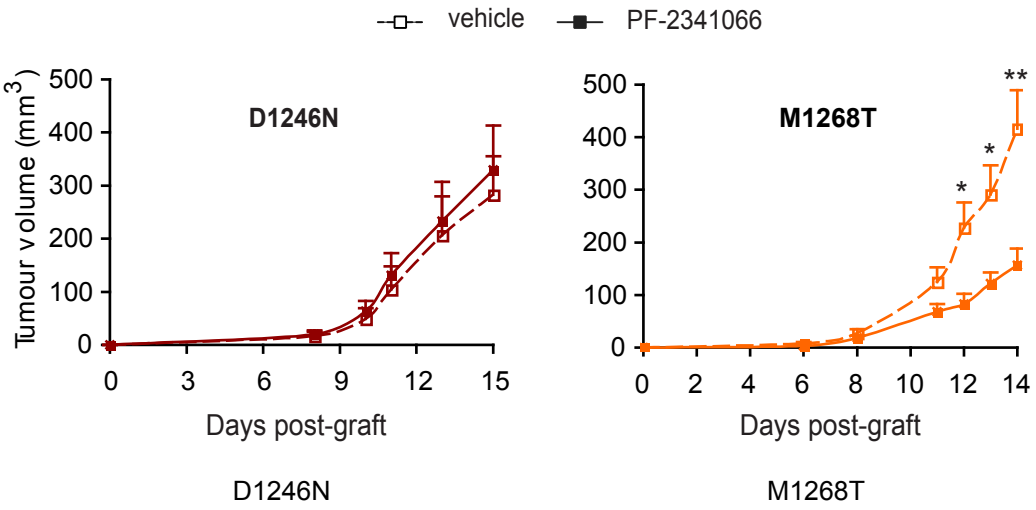


Figure 8: Treatment with Met inhibitors reduces tumourigenesis *in vivo* of M1268T, but not of D1246N, cells
The D1246N and M1268T cells were injected subcutaneously into nude mice (minimum of 5 mice per group). The graphs show the tumour growth curves of the different cell lines over time (mean tumour volume ± SEM). Tumours were measured daily and treated by topical application of either DMSO or PF-2341066 (0.2µM) daily over the surface of the tumour, once tumours reached a volume of 30-50mm^3.

2) Endocytosis inhibition reduces oncogenicity of Met mutants D1246N and M1268T *in vitro* and *in vivo*.

As previously mentioned, the D1246N and M1268T Met mutants have increased levels of endocytosis. The resulting increased accumulation of these constitutively active Met mutants on endosomes leads to persistent endosomal signalling and cell transformation. I present here the experiments I performed during the study published (Joffre, Barrow et al, Nat Cell Biol 2011), unless otherwise specified, which aimed at understanding the mechanism of increased endocytosis of these mutants. The purpose of this was that through inhibiting endocytosis, we were hoping to reduce the accumulation of mutant Met on endosomes and, consequently, reduce cell transformation *in vitro* and *in vivo* tumourigenesis.

a) M1268T Met internalisation is not dependent on Met phosphorylation

One obvious possible reason for the increased endocytosis of the Met mutants is due to their increased level of phosphorylation. Usually an RTK becomes phosphorylated upon ligand binding and this is then followed by receptor internalisation. Together with Carine Joffre we performed a biotinylation internalisation assay to follow Met internalisation from the plasma membrane over 15 minutes in the absence or presence of the Met inhibitor PHA-665752. We observed that the M1268T mutant is constitutively internalised without the need for ligand binding **(Figure 9)**. Surprisingly, however, a decrease in the activation status of the M1268T mutant, through treatment with PHA-665752, did not affect the levels of Met endocytosis

(Figure 9). This demonstrates that the increased endocytosis is not a consequence of the increased phosphorylation of the M1268T mutant.

b) Met internalisation is clathrin, Grb2 and c-Cbl dependent

We then tried to determine some of the mechanisms involved in the increased endocytosis of the Met mutants. As clathrin dependent endocytosis has been shown to be a major route of Met internalisation, I investigated this pathway. I performed knockdown of clathrin heavy chain (CHC) by siRNA and Dr. Carine Joffre developed cells with a stable knockdown using shRNA. I have performed Western blots to check the levels of CHC in cells knocked-down by si- or shRNAs and observed that a good knockdown of CHC was achieved in both cases (**Figure 10A**).

Subsequently the ability of clathrin knockdown to prevent Met internalisation was investigated. I should mention here that both siCHC and chCHC were shown to prevent both constitutive and HGF-bound Met internalisation very effectively and here I present only the data that I personally obtained. Initially, I confirmed that the knockdown of CHC prevented the internalisation of M1268T Met through performing a biotinylation internalisation assay over 15 minutes of incubation at 37ºC. The quantification of this biochemical assay showed Met endocytosis was reduced by 98% upon CHC knockdown by siRNA over control siRNA (**Figure 10B**). This inhibition of Met internalisation following clathrin knockdown was also observed by immunofluorescence, when cells were stimulated with HGF* [157] (**Figure 10C**). After 15 minutes, HGF* was observed in the cytoplasm of cells transduced with control shRNA,

while the majority of HGF-alexa555 remained at or near the plasma membrane in cells transduced with CHC shRNA, confirming that clathrin knockdown prevents Met internalisation **(Figure 10C)**. Importantly, I observed by Western blot that clathrin knockdown did not affect Met phosphorylation, indicating that the Met mutants' activation is not dependent on their internalisation, or Met expression levels **(Figure 10D)**.

c-Cbl is an E3 ubiquitin ligase and adaptor protein which plays a role in RTK and, in particular, Met internalisation[65]. Grb2 is an adaptor protein that binds directly to Met and has been shown to be involved in regulating clathrin dependent endocytosis of receptor tyrosine kinase receptors through acting as a scaffold for c-Cbl binding [65]. Furthermore, it has been proposed that both the D1246N and M1268T mutants are associated with Grb2[286, 287]. We therefore hypothesised that c-Cbl and Grb2 are involved in the increased endocytosis of the Met mutants.

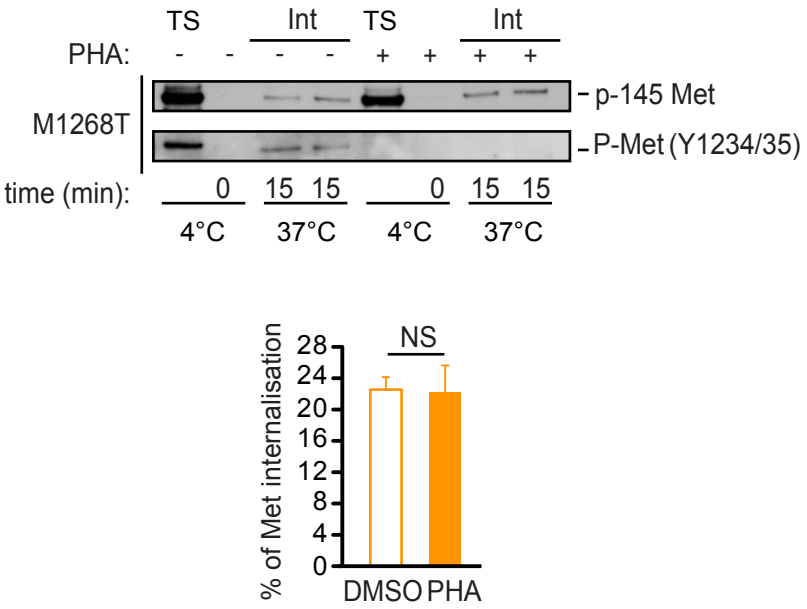


Figure 9: M1268T Met undergoes constitutive internalisation independent of its phosphorylation status.
Biotin internalisation assay. Cells expressing M1268T Met mutant were pretreated (+),or not (-), with PHA-665752 for 30 minutes, surface biotinylatedand then incubated at 37°C for 15 minutes with or without PHA-665752. The biotin was then cleaved, and the remaining biotinylated Met was analysed by Western blotting with anti-Met or anti-phospho-Met (Y1349) antibodies.TS = total surface Met. Int = internalised Met. The graph shows the percentage of Met internalisation following treatment with DMSO and PHA-665752. Data are mean ± SEM (n=3). NS=not significant.

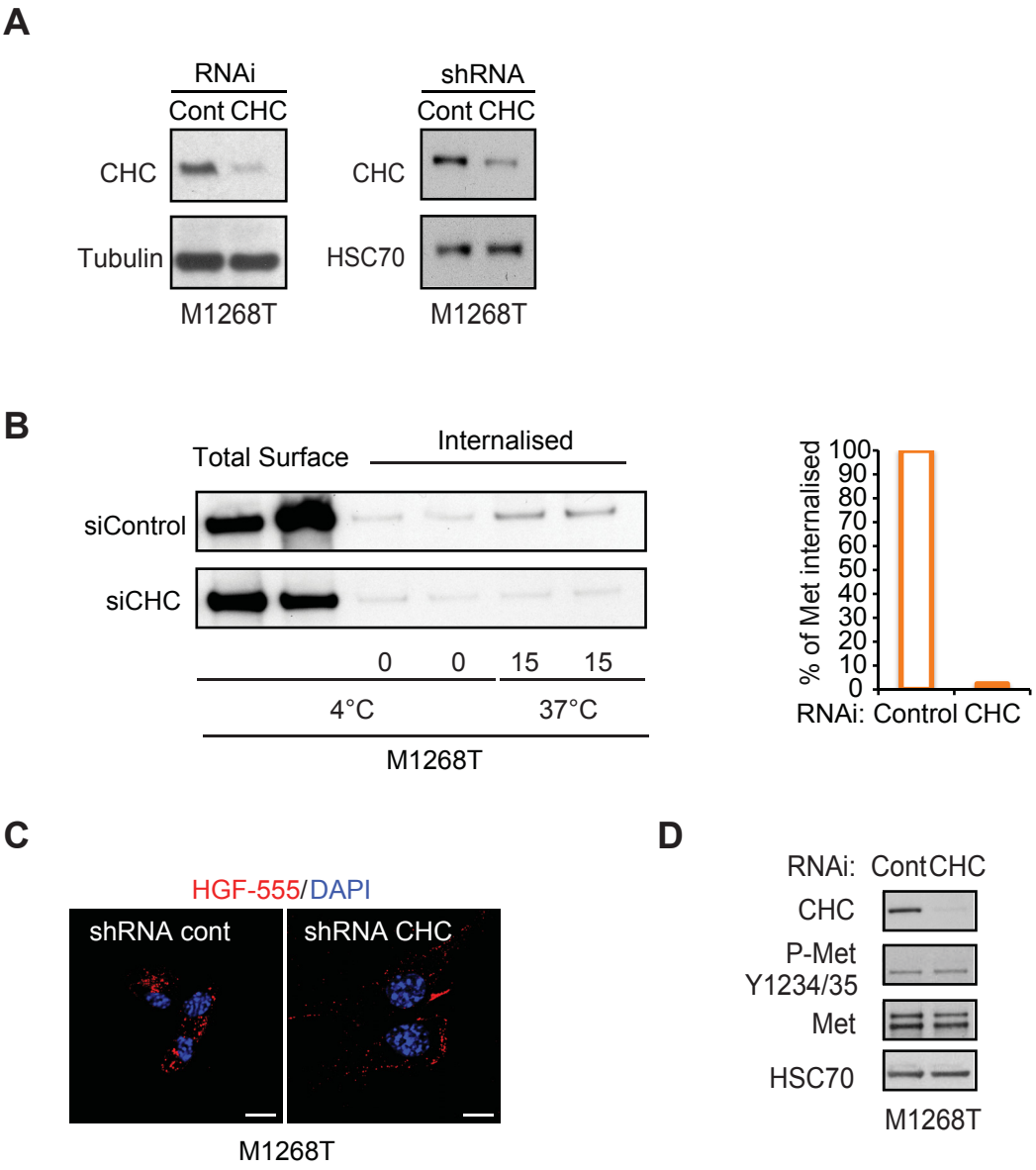


Figure 10: Clathrin knockdown inhibits Met mutant internalisation without affecting the activation status of Met
(A-D) M1268T cells were knocked down for clathrin heavy chain (CHC) or non-silencing control by siRNA transfection or shRNA transduction as indicated. **A)** Western blots for CHC and the loading control tubulin. **B)** Biotinylation internalisation assay. Cells were surface biotinylated and then incubated for 15 minutes to allow internalisation of surface proteins. The biotin was then cleaved from the surface. The remaining biotinylated proteins were pulled down using Strepdavidin beads and analysed by Western blot for Met expression. The graph shows the quantification of Met internalisation by densitometry normalised to the siControl. Data are mean (arbitrary units) ± SEM. **C)** Confocal sections of cells transduced with control or CHC shRNA. The cells were stimulated with HGFAlexa555 (red), fixed and stained for DAPI (blue). Scale bars: 10µm. **D)** Western blots for CHC, phospho-Met, Met and the loading control HSC70.

I achieved a good knockdown of both c-Cbl and Grb2 by siRNA using a nucleofactor transfection method (**Figure 11AB**). Furthermore, as observed with CHC knockdown, Grb2 or c-Cbl knockdown did not affect Met phosphorylation or Met expression levels (**Figure 11AB**). Together with Dr. Carine Joffre we showed, however, that the endocytosis of the M1268T mutant was greatly reduced, using biotinylation internalisation assays (**Figure 11C**). In order to verify the specificity of these mechanisms of internalisation as regards to Met, Transferrin-546 uptake (the ligand of the constitutively recycling non-RTK Transferrin receptor) was monitored as a control. Both Dynamin inhibition (using Dynasore) and CHC knockdown inhibited Transferrin-546 internalisation, however upon c-Cbl or Grb2 knockdown Transferrin-546 uptake could still be observed **(Figure 11D)**, consistent with the fact that c-Cbl and Grb2 are required for RTK internalisation only. Furthermore, I did not detect EGFR expression in these cells, removing the possibility of any effect of inhibition of EGFR internalisation, which also can be regulated by Grb2 and c-Cbl[288] **(Figure 11E)**.

Finally, in order to be more specific, we sought to impair Grb2 binding to the Met mutant. To do this, the M1268T Met mutant was additionally mutated on N1358H, in the +2 position of Y1356 in the Met docking site, in order to abolish the Grb2 consensus sequence without obstructing other molecules binding to Met[289]**.** For this, a human Met Wt cDNA (hWt) was mutated on M1268T (hM1268T) or on both M1268T and N1358H (hM1268T/N1358H), by Dr. Veronique Calleja; these constructs were subsequently sequenced by Dr.

Ludovic Menard. I then transfected these respective constructs into NIH3T3 cells and made stable cell lines using G418 (800μg/ml) for selection. Western blots of the lysates from the stable cell lines demonstrated that the hM1268T mutation triggered an enhanced Met phosphorylation (**Figure 12A**). Furthermore, together with Dr Ludovic Menard, we observed a significantly increased colocalisation of hM1268T with EEA1 (**Figure 12B**), and thus internalisation, versus hWt; confirming results obtained with murine Met forms. Moreover, while Met phosphorylation was maintained in the double mutant (**Figure 12A**), its colocalisation with EEA1 was significantly reduced as compared to the single mutant ($p<0.05$) and was similar to Wt Met (**Figure 12B**), demonstrating its reduced endocytosis.

Thus, these results show that clathrin, c-Cbl or Grb2 expression, as well as the Grb2 consensus sequence on Met mutants, are required for the endocytosis of the Met mutants, such that their knockdown (or mutation in the case of the Grb2 consensus sequence) provide appropriate methods to block Met internalisation.

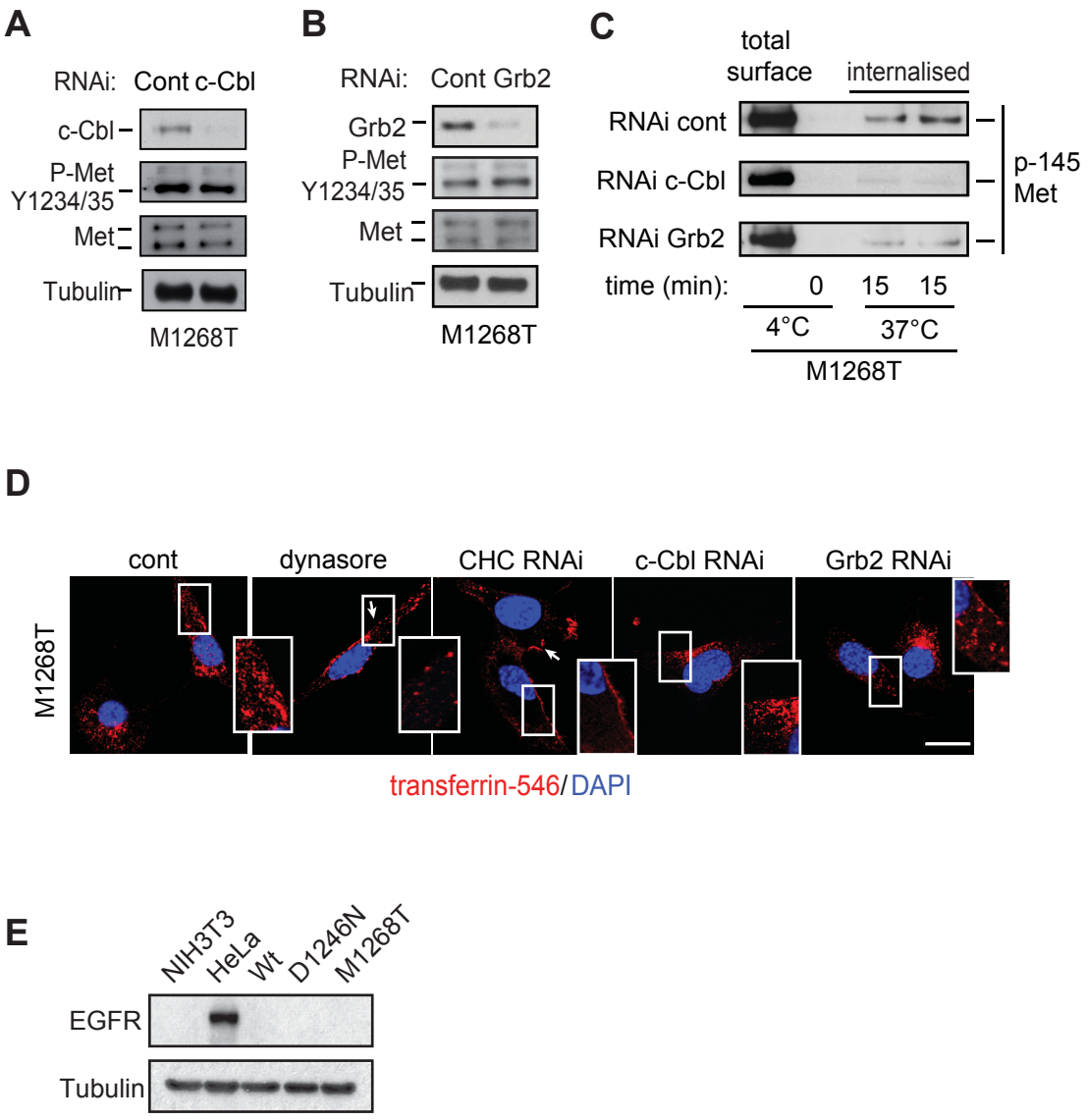


Figure 11: Met mutant internalisation is inhibited more specifically by Grb2 or c-Cbl knockdown

AB) M1268T Met expressing cells were transfected with control, c-Cbl or Grb2 RNAis. Western blots for **A)** c-Cbl, **B)** Grb2, phospho-Met (Y1234/35), Met and HSC70 or tubulin are shown. **C)** Biotin internalisation assay on M1268T Met mutant expressing cells following transfection with control and c-Cbl or Grb2 RNAis. **D)** Representative confocal sections of M1268T expressing cells transfected with RNAi control (Cont), treated with Dynasore or transfected with CHC, c-Cbl or Grb2 RNAi. Cells were incubated with transferrin-546, fixed and stained for DAPI (blue). Arrows indicate transferrin retained at the plasma membrane. Scale bars: 10µm. **E)** Western blots for EGFR and tubulin from the indicated cell lysates are shown.

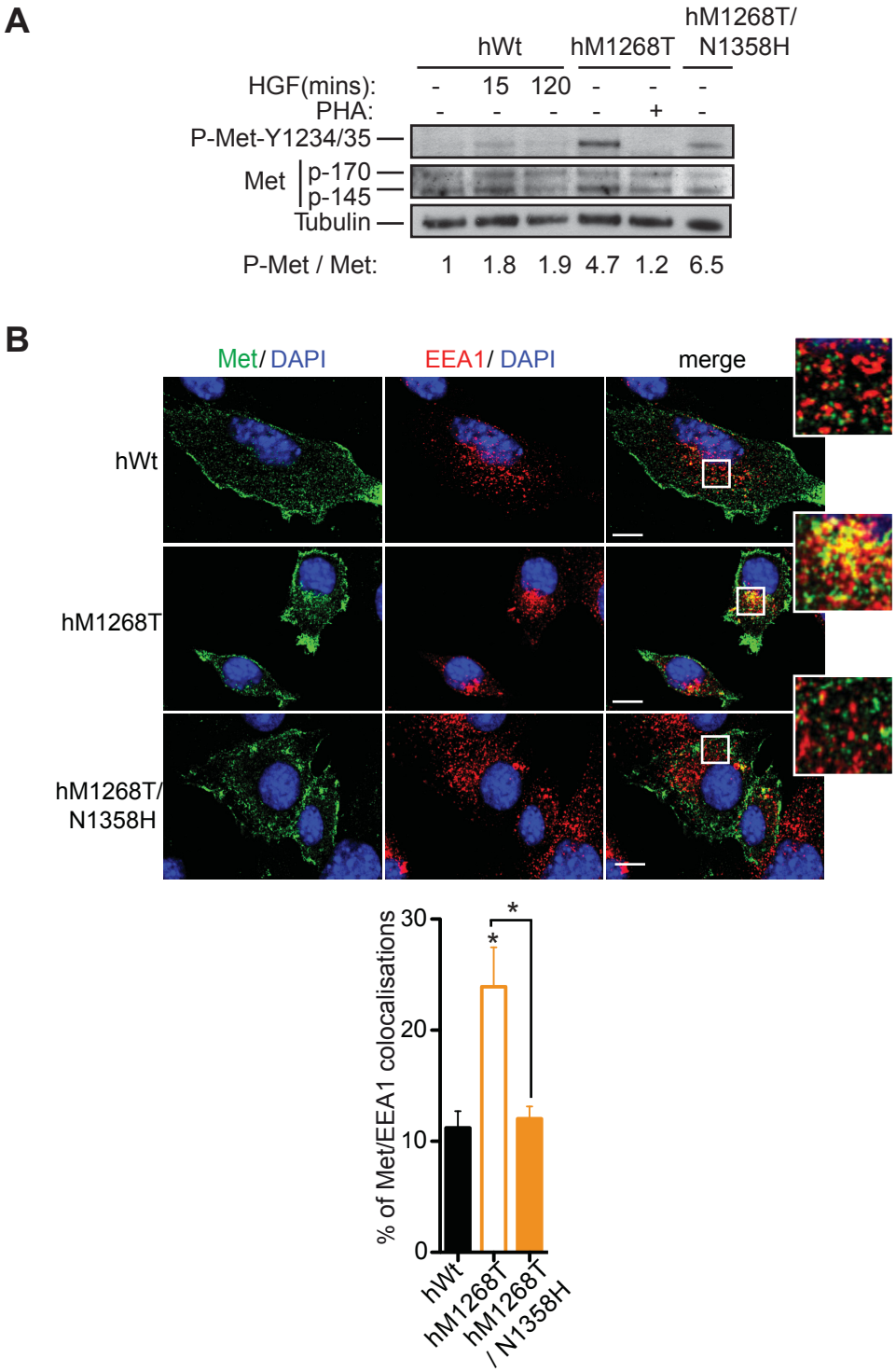


Figure 12: A mutation in the Grb2 binding site of hM1268T (hM1268T/ N1358H) decreases the accumulation of Met on endosomes

A) Met and tubulin Western blots from cells expressing human Met forms, hWt, hM1268T and hM1268T/N1358H, stimulated (+) or not (-) with HGF or PHA-665752. Numbers below are the densitometric values for phospho-Met/ Met. **B)** Confocal sections of cells expressing human Met constructs, hWt, hM1268T and hM1268T/N1358H, pre-treated with cycloheximide for 4 hours and stained for Met (green), EEA1 (Early Endosome Antigen 1, red) and DAPI (blue). Scale bars: 10µm. The graph (below) represents the percentage of colocalisation. Data are mean (arbitrary units) ± SEM (n=3). *p<0.05.

c) Inhibition of Met endocytosis reduces *in vitro* and *in vivo* tumourigenicity of the oncogenic Met mutants.

Having found ways to inhibit the endocytosis of the Met mutants, we investigated whether the endosomal localisation of active Met mutants was required for their *in vitro* and *in vivo* tumourigenicity.

First, I looked at the effect of endocytosis inhibition on the organisation of the Met mutants' actin cytoskeleton. Reducing Met mutant accumulation on endosomes using c-Cbl or Grb2 knock-down led to a significant restoration of actin stress fibres to a similar degree as Met knockdown in both the D1246N and M1268T cells ($p<0.05$), while there was no effect on Wt cells **(Figure 13AB)**.

This suggested that there would consequently also be a reduction in the migration of Met mutant cells. Indeed, in Transwell migration assays, c-Cbl or Grb2 knockdown greatly reduced the high level of cell migration observed in both mutant expressing cells, again to a level similar to that observed with Met knockdown (**Figure 13C**), while there was no effect on Wt cell migration. The M1268T mutant expressing cells transduced with shCHC also had a great reduction in cell migration. Furthermore, cells expressing Met hM1268T/N1358H were significantly less migratory as compared to the hM1268T cells **(Figure 13C)**.

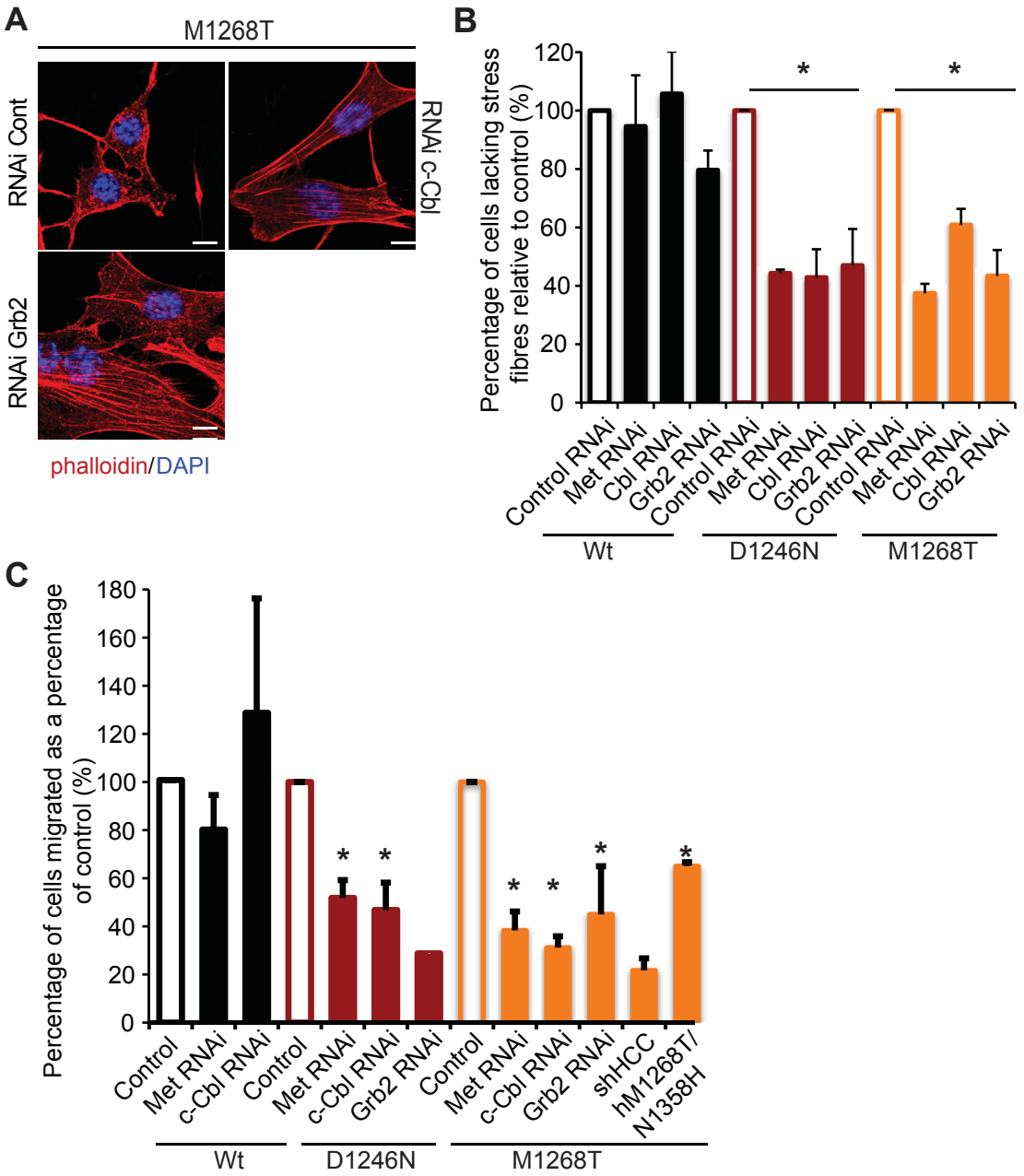


Figure 13: Inhibition of Met mutant internalisation reduces cell migration **A)** Confocal sections of M1268T Met expressing cells transfected with control, c-Cbl or Grb2 RNAi, then stained with Cy3-phalloidin (red) and DAPI (blue). Scale bars: 10μm. **B)** Percentage of Wt, D1246N or M1268T Met expressing cells lacking stress fibres when transfected with Met (n=3), Grb2 (n=3) or c-Cbl (n=3) RNAi. Data are normalised to cells transfected with control RNAi. Data are mean (arbitrary units) ± SEM. **C)** Percentage of Wt, D1246N or M1268T Met expressing cells that have migrated through Transwells when transfected with Met (n=3), c-Cbl (n=3) or Grb2 RNAi (M1268T: n=3, D1246N: n=1), or transduced with CHC shRNA (n=2). The last column represents the percentage of migration of cells expressing hM1268T/N1358H human Met (n=3). Data are normalised to appropriate controls (DMSO, RNAi control, shRNA control, and cells expressing hM1268T Met respectively). Data are mean (arbitrary units) ± SEM, each experiment was performed in triplicate. *p<0.05.

Carine Joffre previously showed that the loss of actin stress fibres observed in the Met mutants was a consequence of Rac1 activity, as knockdown of Rac1 **(Appendix Figure 3A)** or treatment with the Rac1 inhibitor NSC23766 (data not shown)[159], restored the stress fibres and also consequently reduced M1268T cell migration. She also showed that Rac1 was highly activated in the D1246N and M1268T mutant Met containing cells, through doing a GST pull down assay **(Appendix Figure 3B)**, and furthermore Rac1 activation could be decreased by treating M1268T cells with PHA-665752 (100nM) **(Appendix Figure 3C)**. This effect of PHA-665752 could also be observed as a decrease of Rac1 staining at the plasma membrane[159]. Rac1 has been shown to be activated on endosomes from where active Rac1 relocates to the plasma membrane to carry out its role in actin remodelling[158]. Interestingly, Dr. Ludovic Menard observed Rac1-GTP colocalisation with M1268T Met on endosomes.

All together these results strongly indicated that Met mutants activate Rac1 from endosomal compartments, leading to the loss in stress fibres and enhanced cell motility.

Thus we went on to investigate this pathway, using the presence of strong Rac1 staining (by immunofluorescence) at the plasma membrane as a read out of Rac1 activity, as shown by the fact that treatment with PHA-665752 reduces plasma membrane staining of Rac1. In Met mutant expressing cells, there was an increased percentage of cells with Rac1 present at membrane protrusions in D1246N and M1268T cells (64 and 66% respectively) compared to Wt cells (40%, $p < 0.05$) (**Figure 14AB)**. Blocking Met internalisation using c-Cbl or Grb2 knock down led to a significant reduction

in the percentage of both D1246N and M1268T mutant expressing cells that had Rac1 present at membrane protrusions ($p<0.05$), to the same level as Met knock down. Furthermore, the reduction observed resulted in the percentage of cells with Rac1 at membrane protrusions reaching the same level as Wt expressing cells, in which no effect of Met, c-Cbl or Grb2 knockdown was detected.

The hM1268T Met mutant expressing cells were found to behave in the same way as the mouse M1268T Met expressing cells. Cells expressing hM268T Met had a significantly higher percentage of cells with Rac1 at membrane protrusions compared to the hWt cells, as quantified by Dr. Ludovic Menard **(Figure 14C)**. This observed increase in Rac1 at membrane protrusions in hM1268T cells was significantly reduced by treatment with PHA-665752 ($p<0.01$), confirming that this phenotype is dependent on the Met mutation. Moreover, hM1268T/N1358H Met expressing cells had a significantly reduced percentage of cells with Rac1 localisation at membrane protrusions, to a similar level as that observed in hWt cells ($p<0.01$) (**Figure 14C**), confirming that Met mutant endocytosis is required for the full activation of the Rac1 pathway.

These results indicate that the Rac1 activation, induced by the accumulation of the active Met mutants on endosomes, and consequent loss of actin stress fibres, can be greatly reduced by blocking Met endocytosis, using a panel of endocytic blocking tools.

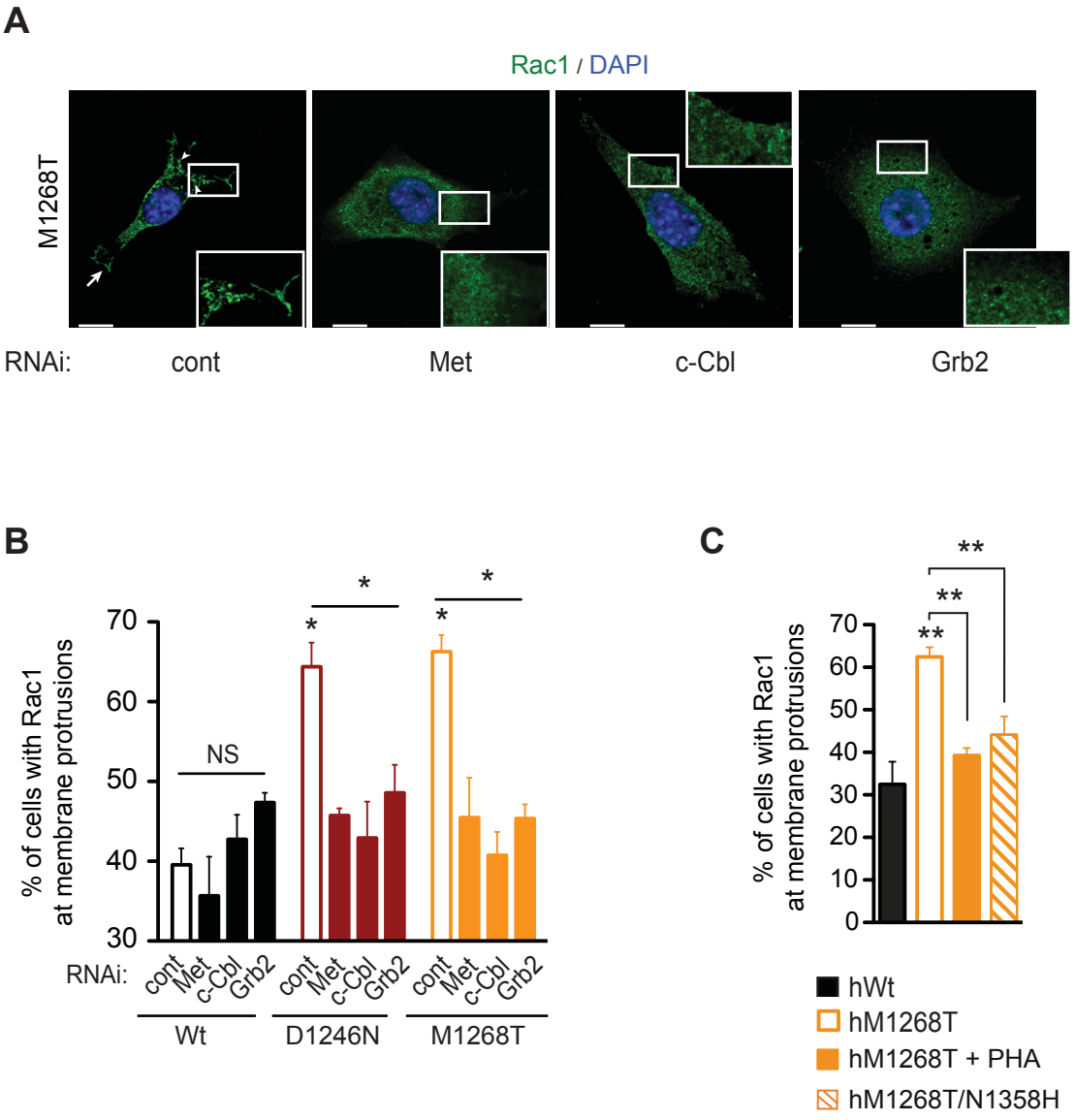


Figure 14: Inhibition of mutant Met internalisation reduces Rac1 localisation at membrane protrusions

A) Confocal sections of M1268T Met expressing cells transfected with control, Met, c-Cbl or Grb2 RNAi, fixed and stained for Rac1 (green) and DAPI (blue). **B)** The graph represents the percentage of Wt, D1246N or M1268T cells with marked Rac1 staining present at membrane protrusions when transfected with control RNAi, Met RNAi, c-Cbl RNAi or Grb2 RNAi. Data are mean ± SEM (n=3). **C)** Percentage of cells expressing human Met forms, hWt, hM1268T and hM1268T / N1358H, that display Rac1 at membrane protrusions. hM1268T were untreated or treated with PHA for 90 minutes. Data are mean ± SEM (n=3). *p<0.05; **p<0.01; NS=not significant.

Using the same endocytic blockers, I then carried out soft agar assays to assess the ability of the cells to undergo anchorage independent growth following inhibition of Met internalisation. After transfection with c-Cbl or Grb2 RNAi, the cells were grown in soft agar for 6 days. The use of these methods to block Met endocytosis led to huge reduction in total area of soft agar colonies of both D1246N ($p<0.05$) and M1268T ($p<0.01$) Met mutant expressing cells to a similar level as measured in Wt cells (**Figure 15A**). Importantly, no effect was observed on Wt cells (**Figure 15A**). The number of colonies was also significantly reduced (**Figure 15B**). Furthermore, hM1268T/N1358H Met expressing cells had a significant reduction (60%) in colony number compared to hM1268T cells (**Figure 15B**).

These results indicate that blocking endocytosis can reduce the transformation of Met mutant expressing cells and may consequently reduce tumour growth *in vivo*.

I then went on to block Met endocytosis, using these newly acquired methods, and investigate whether the endosomal accumulation of the Met mutants is required for tumour growth. To achieve this, the M1268T cells transduced with shControl and shCHC were injected subcutaneously (by Professor Ian Hart) into nude mice and I measured the tumour sizes daily. Interestingly, the tumours formed by the clathrin-depleted (shCHC) cells had a significant (50%) reduction in size ($p<0.05$) compared to the shControl tumours (**Figure 16A**). Surprisingly, but consistent with *in vitro* results (**Figure 10D**), the tumours derived from the shCHC cells had no decrease in Met phosphorylation as compared to shControl tumours (**Figure 16B**). This

suggests very strongly that the decrease in tumour growth was due purely to the change in Met mutant localisation rather than due to any effect on the activation status of Met.

In order to confirm the results obtained by clathrin knockdown, I treated Wt and M1268T expressing tumours with dynole 34-2, a novel, highly-specific, inhibitor of dynamin, which inhibits both clathrin-dependent and clathrin-independent mechanisms of internalisation[290]. Wt and M1268T mutant cells were allowed to form tumours in athymic nude mice (Injected by Professor Ian Hart). I measured the tumour sizes daily and once tumours reached $50mm^3$, I topically applied on the tumours either DMSO or dynole 34-2. Treatment with dynole 34-2 led to a significant decrease in tumour growth, the tumour sizes being $450mm^3$ in the control and $320mm^3$ in the treated tumours respectively at day 7 of treatment ($p<0.01$) (**Figure 16C**).

In a similar experiment, tumours formed from M1268T mutant cells were treated topically with Dynasore (80μM) leading to a large, significant decrease in tumour growth **(Figure 16D)**.

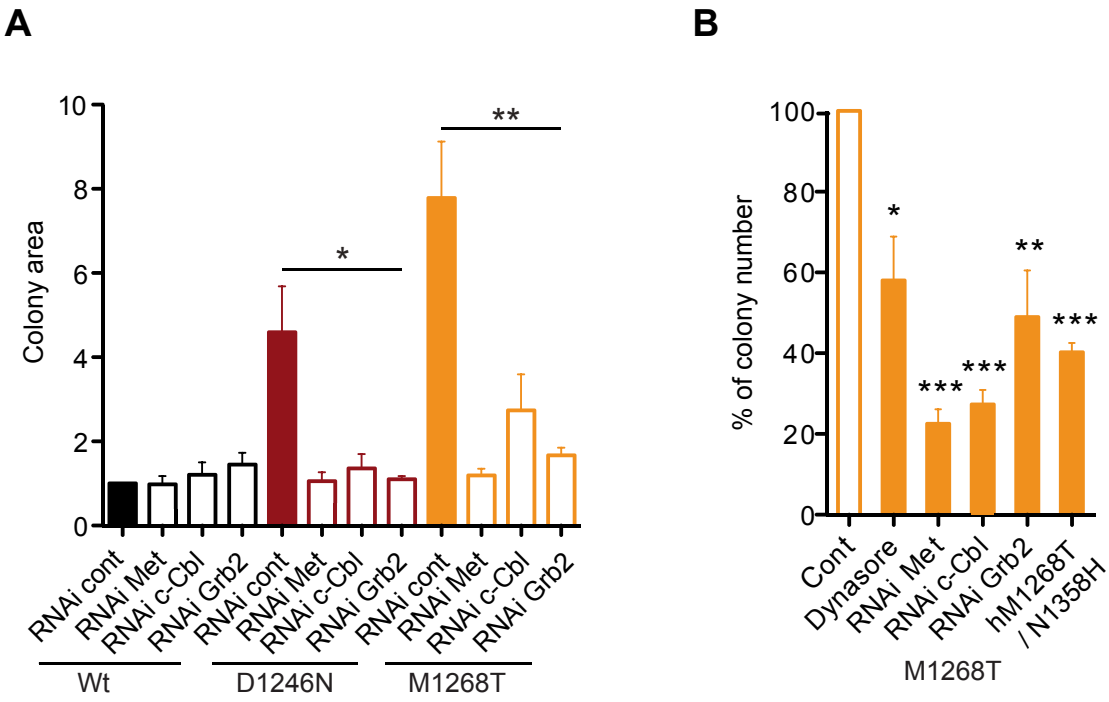


Figure 15: Inhibition of mutant Met internalisation inhibits anchorage independent growth

AB) Cells were cultured in soft agar. Each experiment was done in triplicate. **A)** Wt, D1246N and M1268T cells were transfected with control, Met, c-Cbl or Grb2 RNAis. The graph represents the average colony area. Data are mean (arbitrary units) ± SEM (n=3). **B)** The graph represents the percentage of reduction in colony number formed by cells expressing M1268T Met when treated with Dynasore or transfected with Met, c-Cbl or Grb2 RNAi versus their appropriate controls (DMSO, RNAi cont) (n=3). The last column shows the colony number of cells expressing hM1268T/N1358H Met versus cells expressing hM1268T Met (n=3). Data are mean (arbitrary units) ± SEM. *p<0.05; **p<0.01; ***p<0.001; NS=not significant.

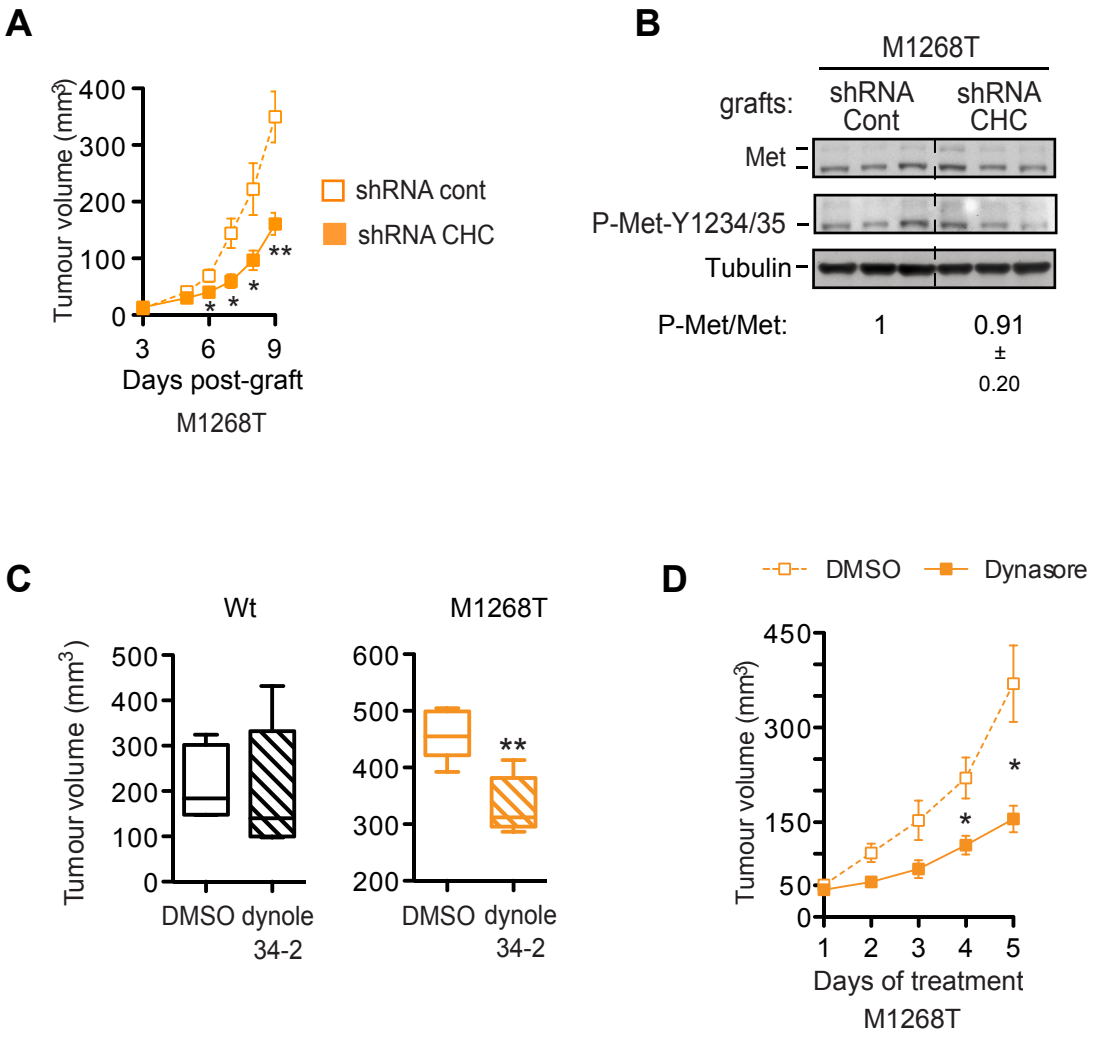


Figure 16: Inhibition of mutant Met internalisation decreases tumour growth *in vivo*

A-D) Cells ($5x10^5$) were injected subcutaneously into the right flank of nude mice (minimum 5 mice per group). When tumours had reached 30-50mm^3, DMSO or inhibitors were applied topically over the surface of each tumour daily. **A)** The graph represents tumour growth curves of M1268T cells transduced with control or CHC shRNA over indicated time. **B)** Western blots for phospho-Met (Y1234/35), total Met and tubulin were performed on tumour samples from 3 different mice per condition (from A). The numbers are the fold increases of phospho-Met/Met for shRNA CHC cells versus shRNA control cells. **C)** Wt and M1268T Met expressing tumours were treated with DMSO or dynole 34-2 (30 µM). The results are represented as box and whisker plots, representing the tumour volume after seven days of treatment. **D)** M1268T Met expressing tumours were treated with DMSO or Dynasore (80 µM) for five days. The graph represents tumor growth curves of the different treatments over the indicated time. Data are mean ± SEM. *p<0.05; **p<0.01.

My results presented here (together with additional results obtained by Carine Joffre, not presented here[159]) confirm that it is the accumulation of the activate Met mutants on endosomes that is the cause of their high tumourigenicity. Indeed, through blocking their endocytosis by different means (clathrin, c-Cbl or Grb2 knock-down, hM1268T/N1358H mutation as well as the dynole 34-2 and Dynasore inhibitors), I was able to reduce Met mutant dependent cell migration, anchorage independent growth and *in vivo* tumourigenicity, without affecting their activation status. Therefore, it is the altered trafficking together with the constitutive activation of the Met mutants that leads to their increased tumourigenicity.

3) Restoration of Met degradation reduces oncogenicity of Met mutants D1246N and M1268T *in vitro* and *in vivo*.

Previous results from our lab by Carine Joffre have demonstrated that Met is degraded in the WT cells over a period of 8 hours, while Met is not significantly degraded in the D1246N and M1268T cells with 86% still remaining after 8 hours[159]. I have managed to replicate these results, confirming that the Wt cells have normal Met degradation over 8 hours HGF, while the D1246N and M1268T cells have little Met degradation **(Figure 17A)**.

My aim was to investigate the mechanisms regulating the stability of the mutants in view of restoring their degradation and consequently cell transformation.

We tested several hypotheses. Firstly, the Met mutants could have a defect in phosphorylation of the Y1003 site, which is necessary for direct binding of c-Cbl, subsequent Met ubiquitination and degradation[88, 89]. Dr. Carine Joffre has investigated this and found that the mutants are in fact constitutively phosphorylated on this site, excluding this hypothesis (supporting data not shown).

Secondly, the Met mutants may alternatively have a defect in their ability to be ubiquitinated. Dr. Carine Joffre and I performed ubiquitination assays and found that the M1268T mutant is, if anything, more ubiquitinated than its Wt counterpart (not shown). Finally, the chaperone protein HSP90, which has been shown to protect various oncogenic RTKs from degradation[291],

including Met[91, 134, 135], could be involved in preventing the degradation of Met mutants. I investigated this possibility.

a) Both Met oncogenic mutants are sensitive to inhibition of HSP90, which restores the degradation of Met mutants.

I first investigated the potential role of HSP90 in maintaining the stability of M1268T and D1246N mutants using a pharmacological inhibitor or RNAi knock down. I monitored Met degradation following time of HGF stimulation by Western blot. Treatment of the M1268T mutant with 100nM 17-AAG (17-allylamino-17-demethoxygeldanamycin), a pharmacological inhibitor of HSP90, significantly restored the degradation of the M1268T mutant Met to that seen in WT Met, such that after 4 hours 63% Met had been degraded ($p<0.01$) **(Figure 17AB)**. I confirmed this by depleting HSP90 expression from M1268T cells. Both HSP90α and HSP90β isoforms were knocked down simultaneously by siRNA using nucleofactor transfection, achieving a good knockdown (**Figure 17D**).

In a similar way to the results obtained following treatment with 17-AAG, HSP90 knockdown restored Met degradation of the M1268T mutant to that of the WT (**Figure 17AB**).

Importantly Met degradation was also restored in the D1246N mutant after 4 hours of HGF stimulation following treatment with 100nM 17-AAG, an interesting result knowing that this mutant is resistant to the small molecule Met inhibitors ($p<0.05$) **(Figure 17AB)**. Importantly, there was a significant decrease in basal Met expression of both of the oncogenic Met mutants following treatment with 17-AAG for 24 hours ($p<0.01$) **(Figure 17C)**,

showing that HSP90 inhibition can restore Met degradation of the Met mutants both upon HGF stimulation and in their ligand-independent activated state.

b) Inhibition of HSP90 reduces D1246N and M1268T dependent anchorage independent growth, however the D1246N mutant requires a higher dose of the HSP90 inhibitor 17-AAG.

I then asked whether the restoration of Met degradation would lead to a reduction in tumourigenicity of the Met mutants. A comparison of the ability of the Wt and M1268T to undergo anchorage independent growth, following either treatment with 17-AAG or transfection with HSP90 RNAi, showed that while HSP90 inhibition had very little effect on Wt cells, it significantly reduced the number and area of M1268T colonies by more than 46% ($p<0.05$), to a level similar to that observed in Wt cells **(Figure 18AB)**.

As the D1246N mutant is resistant to small molecule Met inhibitors it seemed particularly interesting to test HSP90 inhibition on these cells as a potential means for possible therapeutic intervention. However, in soft agar assays, the D1246N cells had only a 29% significant reduction in colony number ($p=0.01$) and a non-significant reduction in colony area following treatment with 100nM 17-AAG (**Figure 18CD**). Nevertheless, transfection with HSP90 RNAi led to a greater decrease both in colony number and area of the D1246N cells (**Figure 18CD**). This led to the idea that perhaps 100nM 17-AAG was not sufficient to efficiently reduce cell transformation triggered by the D1246N mutant and hence I tried higher doses of 150nM and 200nM. Treatment of D1246N cells with 200nM 17-AAG in soft agar assays led to a

small, but significant, 27% decrease in colony number ($p<0.01$) (data not shown) and a larger 50% decrease significant in colony area ($p<0.05$), which was a similar decrease observed following HSP90 knockdown, suggesting that 17-AAG is still specific to HSP90 at this concentration **(Figure 19A)**. Furthermore, a larger degradation of D1246N Met was observed with the doses of 150 and 200nM versus 100nM 17-AAG, both upon HGF stimulation **(Figure 19B)** and under basal conditions (**Figure 19C**), both of which occurred in a dose dependent manner with 200nM being the most effective.

Meanwhile, we also hypothesised that maybe the D1246N mutant is resistant to Met inhibitor due to a particular conformation, which could prevent binding of Met inhibitor to the ATP binding site and this conformation could be maintained by the activity of HSP90. Thus, I tested whether HSP90 inhibition would restore the sensitivity of the D1246N cells to small molecule Met inhibitors. However, a Western blot of D1246N cell lysates showed that Met phosphorylation was maintained in the D1246N cells in the presence of SU11274 together with 17-AAG for 1 hour. Nevertheless, when the treatment with 17-AAG was for 6 hours, an inhibition of Met phophorylation was observed. But this result was also obtained even in presence of 17-AAG alone (**Figure 19D**). This suggests that the activation of the mutant could result from a specific conformation, maintained by the chaperone, though this possibility needs to be examined further in future experiments.

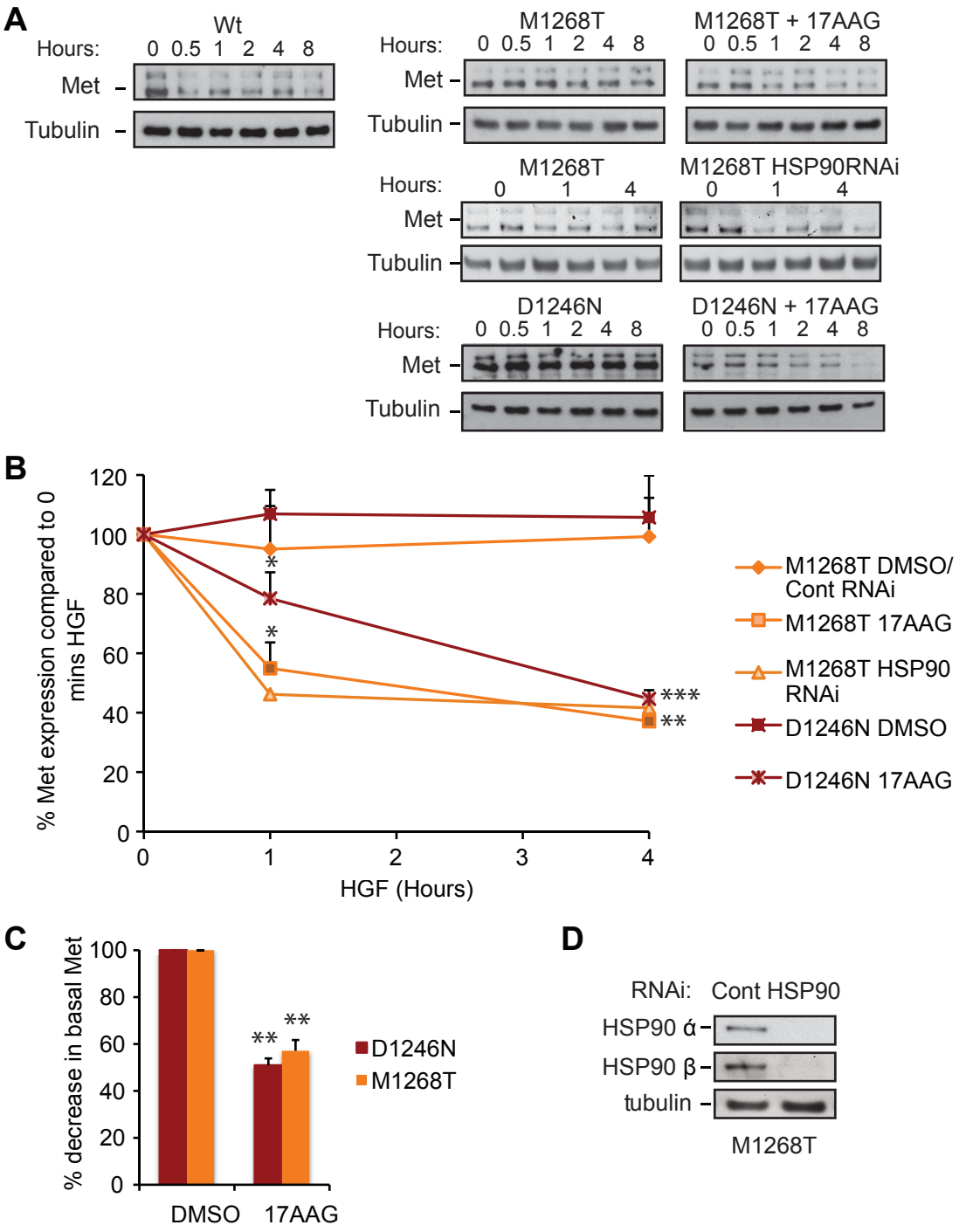


Figure 17: HSP90 Inhibition restores D1246N and M1268T Met degradation

A) Western blots for Met and tubulin on the indicated cells pretreated either with DMSO or 17-AAG (100nM) for 16 hours and treated with HGF (50ng/ml) for indicated times. **B)** Quantification of Western blots using the ImageJ software (M1268T DMSO/RNAi Control n=4; M1268T 17AAG n=3; M1268T HSP90 RNAi n=1; D1246N DMSO and 17AAG n=3). **C)** Quantification of Met levels at 0 minutes HGF following treatment with DMSO or 17AAG for 24 hours. Data are mean (arbitrary units) ± SEM (n=3). **D)** Western blots for HSP90 ά, HSP90 β and tubulin in M1268T cell lysates 72 hours following transfection with control, HSP90 ά and HSP90 β RNAi. *p<0.05; **p<0.01; ***p<0.001; NS=not significant.

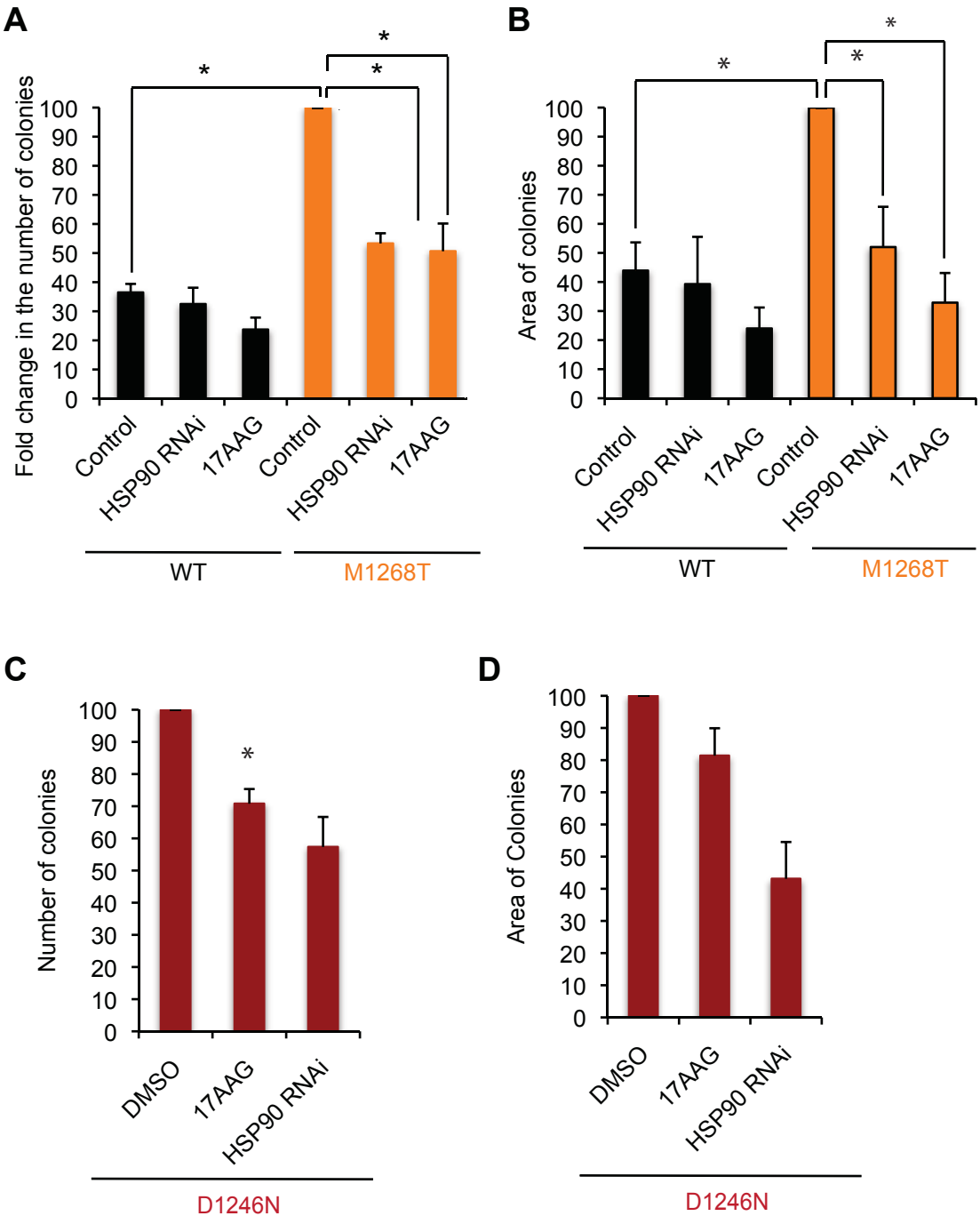


Figure 18: HSP90 inhibition reduces anchorage independent growth of both D1246N and M1268T cells

AB) Results of soft agar assays monitoring the anchorage independent growth of Wt and M1268T cells following treatment with 17AAG (100nM) or transfection with HSP90 RNAi compared to DMSO or Control RNAi controls and normalised to M1268T control. **A)** Number of colonies (n=3). **B)** Colony area (n=3). **CD)** Soft agar assays monitoring anchorage independent growth of D1246N cells following treatment with 17AAG (100nM) or transfection with HSP90 RNAi compared to DMSO or Control RNAi controls. **C)** Number of colonies (n=3). **D)** Colony area (n=3). Data are mean (arbitrary units) ± SEM. *p<0.05.**p<0.01.

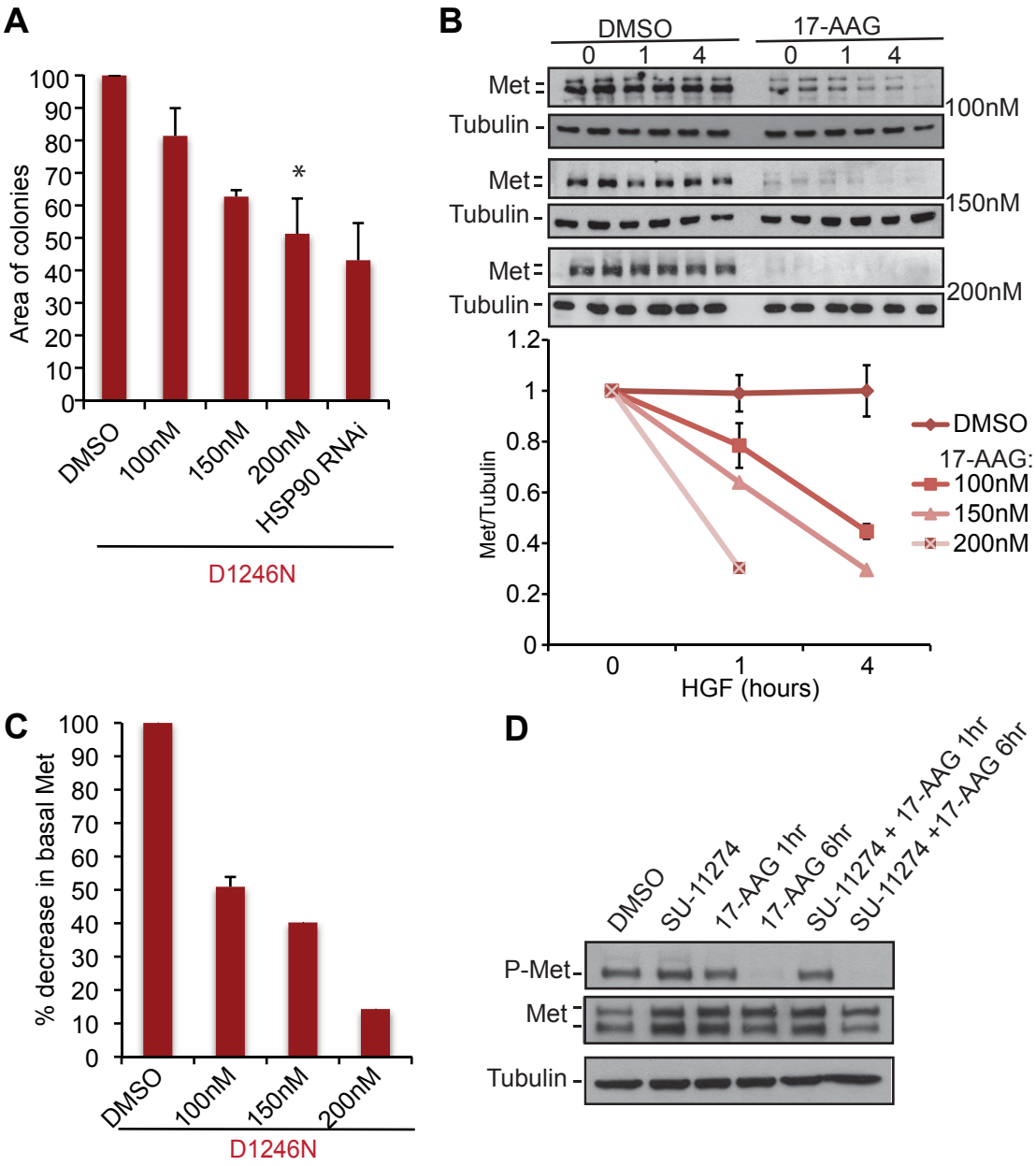


Figure 19: Higher concentrations of 17-AAG restore D1246N degradation more efficiently but specificity is maintained.
A) D1246N cells were cultured in soft agar. The graph shows the average area of colonies measured using ImageJ software. Data are mean (arbitrary units) ± SEM (100nM, 200nM n=3; 150nM and HSP90 RNAi n=2).
B) Western blots for Met and tubulin in D1246N cells stimulated with HGF following treatment with 100, 150 or 200nM 17-AAG and the corresponding DMSO concentration. Bottom panel is the quantification of the western blots. Data are mean (arbitrary units) ± SEM (DMSO n=6; 17AAG 100nM n=4; 17AAG 150nM and 200nM n=1). **C)** Quantification of the percentage decrease in basal Met expression in D1246N cells following 24 hours of treatment with indicated doses of 17AAG. Data are mean (arbitrary units) ± SEM (DMSO/17-AAG (100nM) n=3; 17-AAG (150, 200nM) n=1). **D)** Western blots of phospho-Met, Met and tubulin in D1246N cells treated with DMSO, SU-11274 (2μM) for 1 hour, 17AAG (100nM) for 1 or 6 hours and SU-11274 (2μM) for 1 hour + 17AAG (100nM) for 1 or 6 hours.*=p<0.05.

c) Inhibition of HSP90 reduces M1268T cell migration and *in vivo* tumourigenesis.

While I was establishing the most efficient, yet specific, dose of 17-AAG for the D1246N cells, I continued with the studies on the M1268T mutant. First I asked whether the restoration of M1268T Met mutant degradation would reduce the migratory potential of these cells.

A restoration of stress fibres was observed in M1268T cells following treatment with 17-AAG (100nM) for 1 hour (**Figure 20A**). Subsequently, M1268T cells transfected with HSP90 RNAi had a significant decrease in migration in Transwell migration assays of 41% compared to Control RNAi ($p<0.05$), while there was no effect on cell migration in the Wt cells, confirming that the observed effect is Met mutation dependent (**Figure 20B**).

Following these results, M1268T cells were injected sub-cutaneously into athymic nude mice (by Professor Ian Hart). Once the M1268T tumours reached $50mm^3$, I treated them topically with DMSO (control) or 17-AAG (100nM). Treatment with 17-AAG led to a significant decrease in tumour size ($p<0.05$) (**Figure 20C**). In fact, the same reduction in tumour growth was obtained by blocking endocytosis with Dynasore in an experiment performed at the same time **(Figure 20C)**. This demonstrates that either inhibition of Met internalisation or restoration of Met degradation, both of which reduce the accumulation of the oncogenic Met mutant on endosomes, can inhibit Met mutant tumourigenesis to the same degree.

I am currently investigating mechanisms further using the M1268T mutant as a model. Below, I present the results obtained so far.

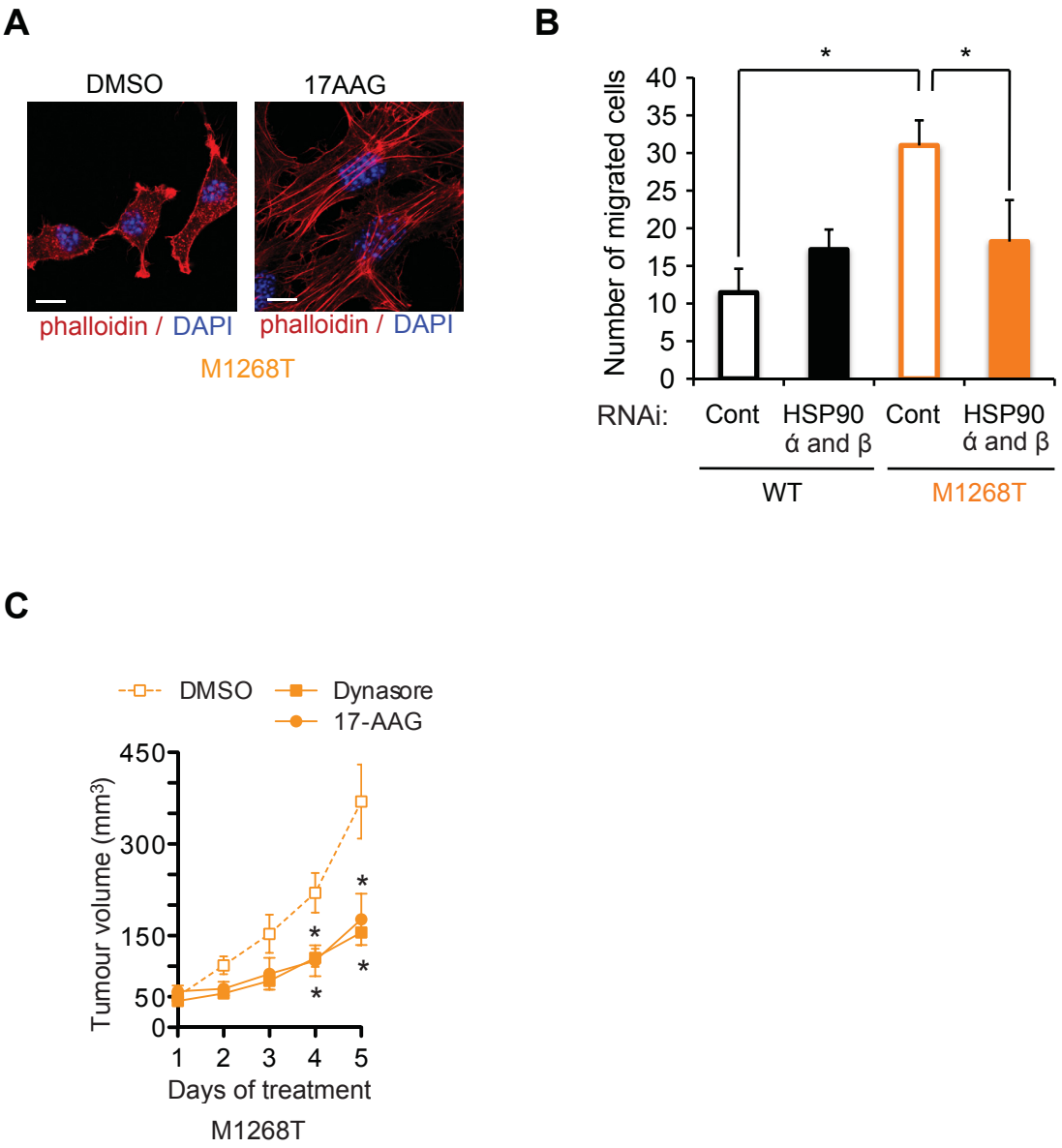


Figure 20: HSP90 inhibition reduces M1268T cell migration and *in vivo* tumourigenesis

A) Confocal sections of M1268T cells treated with DMSO or 17-AAG (0.1μM) for 1 hour and then stained with Cy3-phalloidin (red) and DAPI (blue). Scale bars: 10μM. **B)** Transwell migration assay with Wt and M1268T cells 72 hours following transfection with control or HSP90 RNAi. Data are mean (arbitrary units) ± SEM (n=3). **C)** Growth of tumours *in vivo* using M1268T cells. Cells were injected subcutaneously into nude mice. Tumours were measured daily and treated by topical application of either DMSO, 17-AAG (0.1μM) or Dynasore (80 μM) daily over the surface of the tumour, once they reached 30-50mm^3. Data are mean ± SEM.

d) The HSP90 α isoform is increased in M1268T cells but is not specifically required for cell transformation

Several studies have shown that HSP90 α is the HSP90 isoform that is associated with oncogenic proteins[94, 98]. As I had so far combined RNAis of HSP90 α and β, it seemed interesting to investigate whether a single isoform is required for maintaining mutated Met tumourigenicity. To test this I initially looked at the expression levels of both isoforms in the Wt and M1268T cells by Western blot. While there was no change in HSP90 β expression there was a small, but significant, increase in HSP90 α expression in the M1268T cells compared to Wt cells **(Figure 21A)**. Furthermore, work carried out by Dr. Carine Joffre showed that M1268T Met associates with HSP90 α in Co-IP experiments **(Appendix Figure 4)**.

In order to determine the role of each HSP90 isoform in Met degradation and Met dependent tumourigenesis, I transfected cells with HSP90 RNAi against each isoform individually. Surprisingly, decreased expression of either isoforms **(Figure 17D)** appeared to restore M1268T Met degradation **(Figure 21B)**.

Accordingly, M1268T cell migration was inhibited by decreased expression of either isoform or both together (**Figure 21C**). There was no change in Wt cell migration following HSP90 α knockdown, however decreased expression of both isoforms together led to a significant increase in cell migration of the Wt cells and this correlated with a small increase in cell migration following knock-down of HSP90 β in the Wt cells **(Figure 21C)**. Thus perhaps

decreased expression of the HSP90 β isoform may have a greater adverse effect on normal non-oncogenic cells than the HSP90 α isoform.

However, only decreased expression of both isoforms together decreased the number of M1268T colonies that grew in soft agar. There was no effect on the number of colonies or colony area (data not shown) when each isoform was knocked down individually **(Figure 21D)**. This may have been due to the length of time (6 days) over which this experiment was performed. By the end-point the effect of knockdown of each isoform individually may have been lost, while the knockdown of both isoforms together may be more potent or synergistic such that the effect is maintained for longer.

e) The HSP90 co-chaperone Hop does not appear to protect M1268T mutant Met from degradation

I wanted to investigate which co-chaperone is involved in helping HSP90 protect M1268T Met from degradation. Several co-chaperones have been associated with maintaining oncogenic proteins in a complex with HSP90. The co-chaperone Hop/Stip1 brings together HSP90 and HSP70, which brings the oncogenic client protein to HSP90[104, 105]. Hop seemed to be a promising target as a co-chaperone involved in the M1268T-HSP90 complex, both due to its increased expression in tumours[292] and also due to the fact that its knockdown has previously been shown to decrease Wt Met expression[293].

I investigated the influence of Hop knockdown on M1268T Met degradation. I managed to achieve a good knock-down of Hop in M1268T cells using the

nucleofactor transfection method **(Figure 22)**. However, when I looked at Met expression and degradation upon Hop knock-down I found conflicting results between the two experiments I was able to conduct in the time avaliable. Although in both experiments I saw no restoration of HGF-stimulated Met degradation following Hop knockdown, a large decrease in basal Met expression was detected in the first experiment, while a large increase was detected in the second experiment. The reasons for this are unclear, it may be due to environmental differences or differences in the percentage knockdown between the two experiments and, obviously, further experiments are required to determine which is the true result.

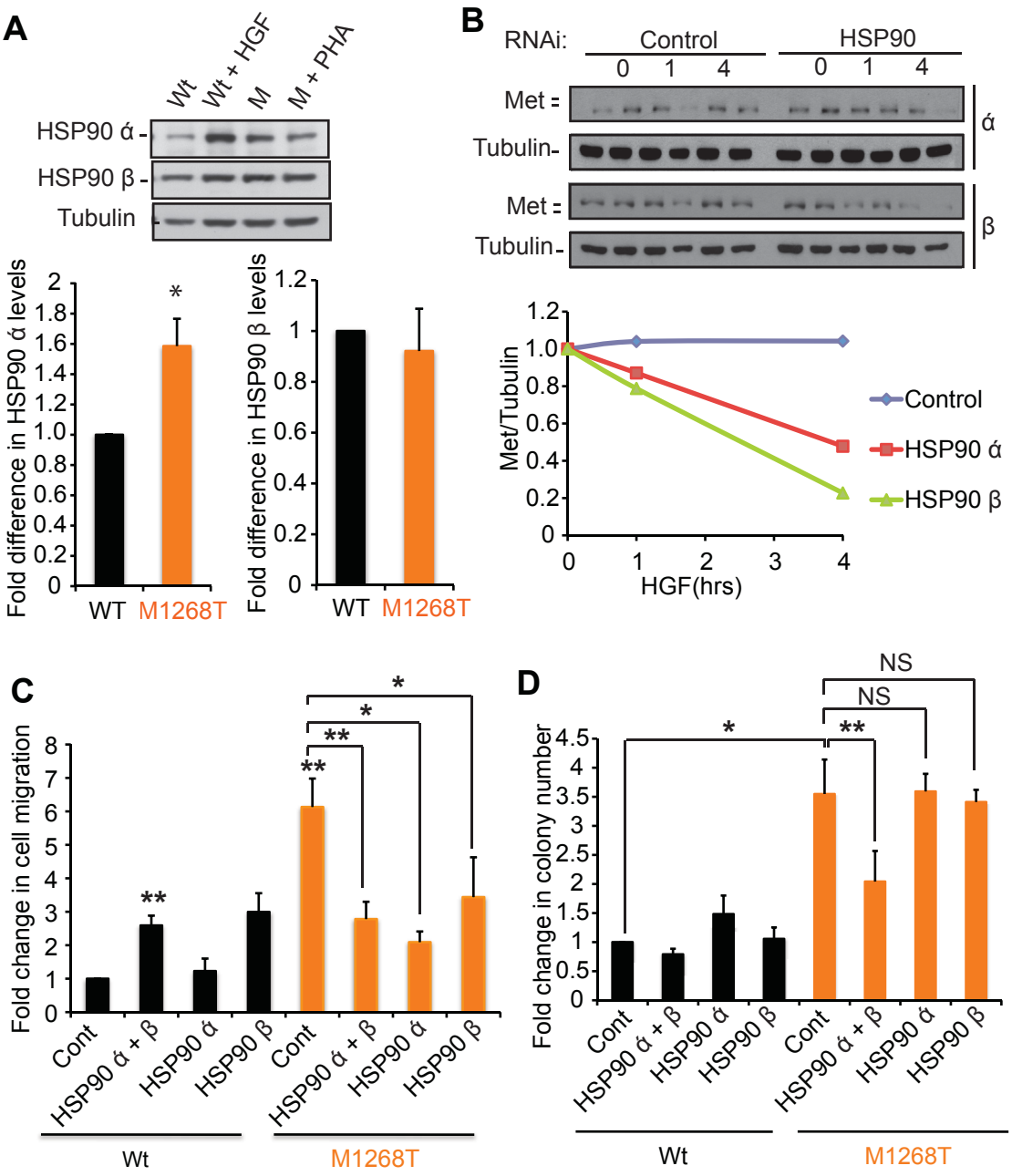


Figure 21: The HSP90 ά isoform is increased in M1268T cells but is not specifically required for migration or anchorage independent growth
A) Western blot for HSP90 ά, HSP90 β and tubulin on Wt +/- HGF and M1268T +/- PHA-665752 (0.1μM) cell lysates. The graphs are quantification of HSP90 ά or β expression by densitometry (left: n=6, right: n=5). **B)** M1268T cells were transfected with Control, HSP90 ά or HSP90 β RNAi. Western blots for Met and tubulin from cell lysates treated with HGF (50ng/ml) for 0, 1 and 4 hours, 72 hours following transfection. The graph shows the quantification by densitometry (n=1). **CD)** Transwell migration assay of Wt and M1268T cells 72 hours following transfection with Control (n=6), HSP90 ά (n=3) or HSP90 β RNAi (n=3) or both HSP90 ά and HSP90 β RNAi (n=6). **D)** Soft agar assay of Wt and M1268T cells 72 hours following transfection with Control, HSP90 ά or HSP90 β RNAi or both HSP90 ά and HSP90 β RNAi (n=3). Data are mean (arbitrary units) ± SEM. *=p<0.05; **=p<0.01; NS=not significant.

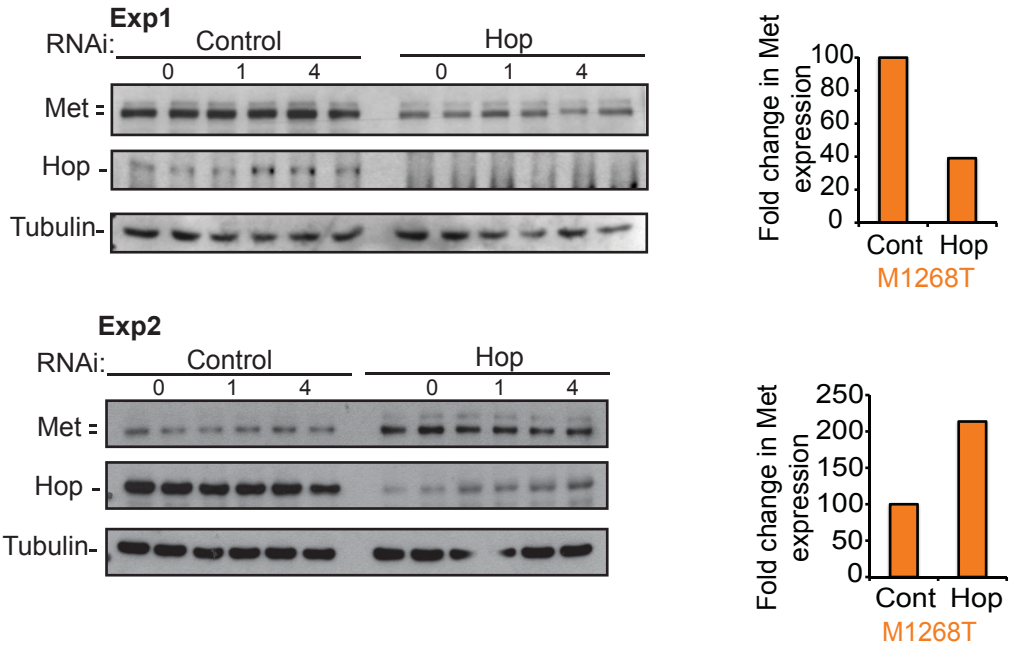


Figure 22: The HSP90 co-chaperone Hop does not appear to be involved in preventing M1268T Met degradation

Western blots for Met, Hop and tubulin in M1268T cells treated with HGF (50ng/ml), 72 hours following transfection with Control or Hop RNAi. Two experiments are shown and the graphs on the right are the quantifications of the basal Met expression (no HGF) for each experiment measured using ImageJ software (arbitrary units).

Overall HSP90 inhibition seems to be a promising treatment option for Met driven cancers. Although the D1246N mutant requires a higher dose of the HSP90 inhibitor 17-AAG to achieve the same level of reduction in Met dependent transformation, I have showed that impairing HSP90 activity or expression re-establishes M1268T Met mutant degradation which reduces its impact on cell migration and *in vivo* tumourigenicity.

DISCUSSION PART II

Understanding Met oncogenic Mutants' signalling and targeting this in tumourigenesis.

In this chapter, I have confirmed that two mutations located within the kinase domain of Met, lead to constitutive phosphorylation of the receptor, which induces cell transformation. I tested three small molecule Met inhibitors on these Met mutant expressing cells and found that while one Met mutant is sensitive to these forms of Met inhibition, another is resistant.

Carine Joffre originally characterised the trafficking of these two naturally oncogenic Met mutants, in NIH3T3 cells. She previously described that they accumulate on endosomes due to constitutive internalisation via dynamin and clathrin dependent mechanisms, and constitutive recycling. Importantly, their location on endosomes is accompanied by their high activation status and she demonstrated that both are required for Rac1 activation, migration and for the *in vivo* tumourigenicity of the Met mutants.

I have extended her work, to further understand the mechanisms of Met mutants' endocytosis, and showed it also is dependent on c-Cbl and Grb2 expression and on Grb2-Met binding. Blocking endocytosis through impairing these mechanisms reduces cell transformation *in vitro* and *in vivo* and importantly does not affect Met activation, confirming that Met localisation together with Met activation are both required for Met mutant dependent tumourigenicity **(Figure 23)**.

Furthermore, Carine showed that the mutants also have a defect in degradation. I found that this is due to the activity of the chaperone protein

HSP90. HSP90 inhibition and/or knock down can restore Met degradation and in doing so can reduce the transforming ability of Met mutant *in vitro* and *in vivo* **(Figure 23)**.

1) <u>Met Oncogenic Mutants: Sensitivity to Met inhibitors</u>

Met mutants are constitutively active and are transforming

I have confirmed that the M1268T and D1246N Met mutants are both more highly phosphorylated than the Wt Met, with the M1268T Met mutant more phosphorylated than Wt Met when stimulated with increasing doses of HGF. The NIH3T3 murine fibroblasts used were chosen as they produce very low levels of HGF[203], suggesting that there is no autocrine loop. This does indeed appear to be the case as there is very little Wt Met phosphorylation, but this can be induced upon HGF treatment. This therefore, indicates that the Met mutant M1268T and D1246N expressing cells are intrinsically and constitutively active. This previously was established by Jeffers et al. where the extracellular domain of Met was replaced by the extracellular domain of the Nerve Growth Factor (NGF) receptor (Trk)[285]. Since NIH3T3 cells do not secrete NGF, the observed phosphorylation and tumourigenicity of the Met mutants was shown to be a result of their constitutive activation rather than the presence of an autocrine loop[285].

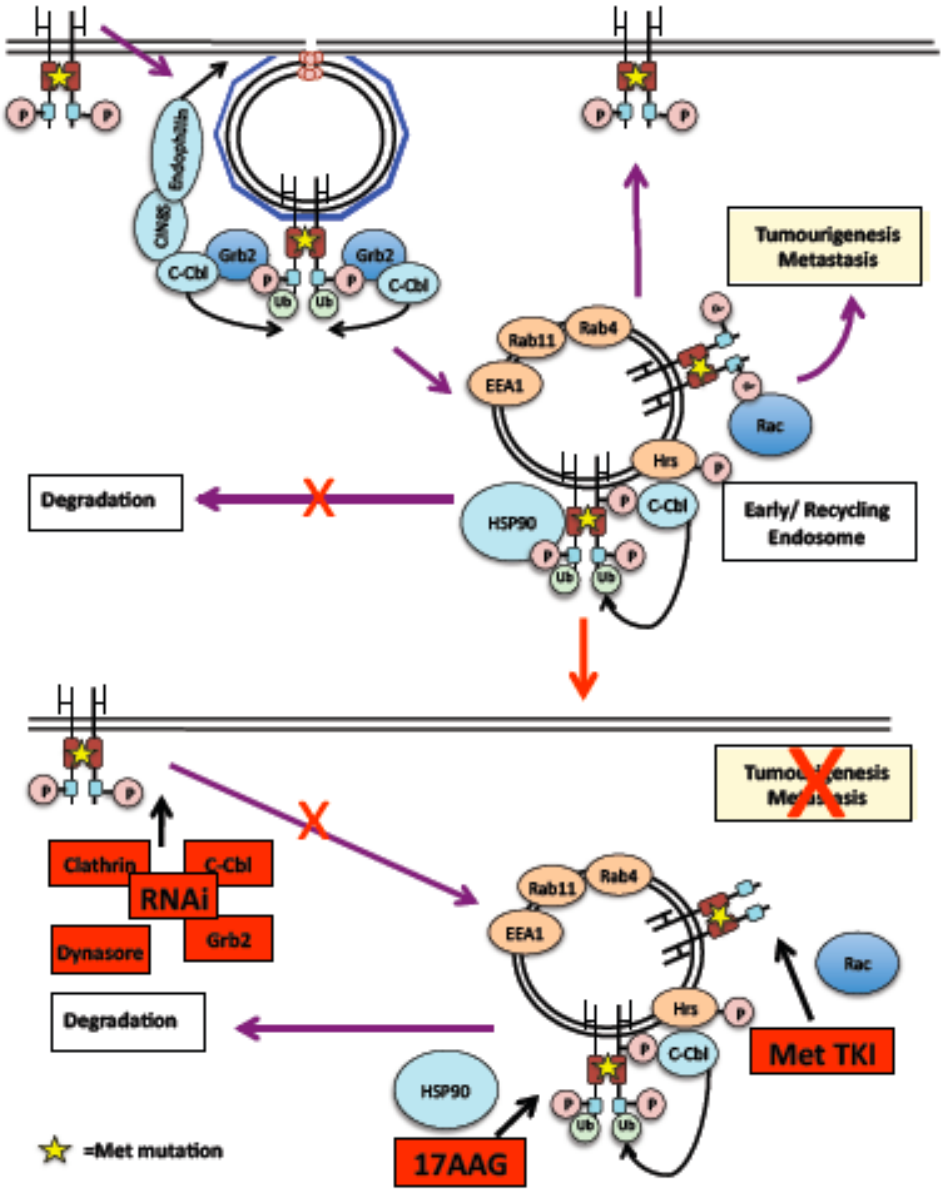


Figure 23: Model of Met oncogenic mutant signalling mechanisms

The D1246N and M1268T Met mutants have a defect in degradation and undergo constitutive internalisation and recycling, leading to transforming signals from endosomes. Met tyrosine kinase inhibitors (TKI) effectively block M1268T Met phosphorylation reducing cell transformation. Inhibition of D1246N and M1268T Met internalisation (using clathrin, c-Cbl, Grb2 RNAi or Dynasore) or restoration of Met degradation (with HSP90 inhibitors or HSP90 RNAi) also reduces cell transformation by both mutants.

As a result of their constitutive activity, I have also observed that these mutants are transforming without the need for ligand binding. They have a transformed morphology, are more migratory, more readily grow in soft agar and form tumours in nude mice more rapidly than cells expressing Wt Met.

Individual Met mutants have different sensitivity to Met inhibitors

Some mutations in EGFR, such as exon 19 deletions and the L858R point mutation, confer an increased sensitivity to TKIs, while others, including insertion mutations into exon 20 or the T790M mutation, confer resistance to tyrosine kinase inhibitors (TKI) [195]. However, very little is known regarding the sensitivity of Met mutants to TKIs.

In my study, three ATP-competitive small molecule inhibitors of Met, PHA-665752 (PHA), SU11274 (SU) and PF-2341066 (PF), were tested on the D1246N and M1268T mutants. The M1268T mutant was highly sensitive to all three inhibitors. This mutant previously has been shown to be sensitive to PF by ELISA assay but no studies on the cell functions and *in vivo* tumourigenesis were done [225].

We have shown that treatment with each inhibitor led to inhibition of M1268T Met mutants' phosphorylation, which consequently reduced cell migration and anchorage independent growth *in vitro*, to the same level as Wt Met, as well as a reduction in tumourigenicity *in vivo*. Furthermore, the reduction in cell migration and anchorage independent growth observed with the three inhibitors was similar to that following Met knockdown using Met RNAi, which had no effect on the Wt Met expressing cells. This confirms that, firstly, the

inhibitors induce near complete inhibition of M1268T Met mutant dependent tumourigenesis and, secondly, there is not a significant autocrine loop occurring in these cells.

The D1246N mutant was, however, resistant to all three inhibitors both *in vitro* and *in vivo*, although at very high doses of PF-2341066, the D1246N mutant exhibited decreased phosphorylation. However, at high doses of PF-2341066, such as 1μM, the drug no longer specifically targets Met, but also inhibits Tie-2, TrkA, TrkB and Insulin receptor activation[225]. In fact, when compared to the M1268T mutant, the D1246N is 100 times less sensitive to PF-2341066 **(Figure 3a)**. Despite the resistance of the D1246N mutant to the various small molecule Met inhibitors, the transforming capability of this mutant was definitely due to the constitutive activation of Met as depletion of Met expression with Met RNAi reduced the stress fibre formation, cell migration and the anchorage independent growth of the D1246N Met expressing cells.

The D1246N and M1268T mutations are located in different regions of the kinase domain, suggesting that this could be the reason for their different sensitivities to TKIs, especially since each of the inhibitors act by binding to Met in a similar way. The M1268T mutation is found in the P+1 loop and destabilises the inhibitory position of the activation loop leading to an active conformation, while the D1246N mutation is located in the activation loop and leads to stabilisation of the active conformation[294]. These structural differences could affect the binding ability of the TKIs. Intriguingly, the

D816Y/V mutation found in the Kit receptor is analogous to D1246N and causes Kit to be resistant to imatinib, but this mutation is sensitive to an alternative and broader TKI, PKC412[295]. In a study by Timofeevski et al., comparing the small molecule inhibitors PF-02341066 and PF-0421709 on a range of oncogenic Met mutations (not including the M1268T and D1246N mutations), it was found that while PF-0421709 was more specific for the Met kinase than PF-02341066 (although they both exhibited high specificity), PF-02341066 could act on a wider range of Met mutations due to a difference in conformation that altered the affinity for inhibitor binding[226]. However, I have shown that the D1246N Met is resistant even to PF-02341066,. But perhaps a broad spectrum TKI against a range of RTKs maybe be effective on the D1246N mutant.

The Met inhibitors tested in this study were all ATP competitive small molecule inhibitors and therefore are likely to bind to Met in the same manner. In fact, Amgen has recently produced the orally available AM7, which binds to a different area of the kinase domain than SU11274 and which has been shown to be effective against the Met mutations that are resistant to SU1124 (not including the D1246N)[296]. So, inhibitors with a different mode of binding may be effective against resistant RTKs. With more time it would certainly have been interesting to test other types of Met inhibitors on these two Met mutations. It is worth noting, however, that some inhibitors preferentially bind non-active or active forms of the receptor. For example, the non-ATP competitive small molecule inhibitor ARQ197, preferentially binds to the non-active form of Met[212]. This information is

valuable knowledge for a patients' treatment strategy as, if the driving oncogene is a constitutively active receptor such as M1268T Met, it is possible that the inhibitor will not efficiently inhibit Met activation.

2) Met Oncogenic Mutants: A role for endocytosis in their oncogenicity

As I have already mentioned, the trafficking of these two Met mutants has previously been characterised by Dr. Carine Joffre, showing that they accumulate on endosomes through constitutive endocytosis and recycling.

Met mutants constitutively internalise independently of Met phosphorylation

Perhaps surprisingly, we have shown that the M1268T Met continues to undergo the same rate of constitutive internalisation when its activation is inhibited in the presence of PHA-665752. It is normally believed that RTKs are activated by their ligand and as a consequence undergo endocytosis. However here I have demonstrated that for the M1268T Met they are two separate events. This has been previously demonstrated for EGFR, where a kinase-dead EGFR (K721A) can internalise and in fact it has been reported that it may actually be receptor dimerisation not activation that induces internalisation[297, 298]. Thus, inhibition of phosphorylation using PHA-665752 may not affect receptor dimerisation. However, to be certain that there is not a small amount of Met phosphorylation remaining following treatment with PHA-665752, another mutation in the kinase domain to establish a kinase dead M1268T Met mutant would be required. Nevertheless, we can conclude

that the increased internalisation of the mutants is not a consequence of their increased activation.

Met mutants internalise via clathrin, Grb2 and c-Cbl dependent mechanisms.

Firstly, I have confirmed that the internalisation of the M1268T Met occurs through clathrin dependent mechanisms, as previously shown for Wt Met stimulated with HGF[82, 157]. Secondly, Carine Joffre additionally found that the mechanism for the increased internalisation of the Met mutants was through a constitutive association with Grb2, which has previously been suggested to occur with the M1268T mutant[286, 287].

Thus, we have shown that the internalisation of the M1268T mutant is dependent on Grb2 binding, which may then bring c-Cbl to Met. In this scenario, c-Cbl will ubiquitinate Met and induce its subsequent internalisation, as has been shown previously to occur with Wt Met stimulated with HGF[65] and indeed we have shown that M1268T Met also requires c-Cbl for internalisation.

Blocking Met endocytosis specifically is a challenge, as it is not really known where there is a consensus sequence that controls Met internalisation. However, c-Cbl and Grb2 knockdown appeared at least to be specific for RTK internalisation and as Met was most probably the major oncogenic trafficking RTK in these cells (EGFR expression was not detected by Western blot), it seemed to be a good approach. More importantly, as we

have proved that the transformation of the Met mutant cells results from Met activity (as inhibited with Met knock down or inhibitor, with no change in the Wt untransformed cells), our functional studies on Met mutant expressing cells using the endocytic blockers were always compared to Wt Met expressing cells.

However to increase specificity, and as we knew that Grb2 was required for M1268T Met internalisation, an additional Met mutant was created; hM1268T/N1358H, which was unable to bind to Grb2 as previously reported by Ponzetto et al.[289]. We were able to show that, in parallel to its reduced internalisation compared to hM1268T, although still highly phosphorylated, it also was less oncogenic, confirming the mutation M1268T triggers Met internalisation in addition to its activation and that mutant M1268T internalisation, together with its activation, drive its oncogenicity. The additional benefit of these human constructs, as opposed to the murine constructs expressed in the cell lines mostly used in our studies[159, 203] is that they contain the human Wt Met both alone and with the mutations; M1268T and M1268T/N1358H. This provides us with the opportunity to study the M1268T mutation in a more physiological model for human cancer, for example by transfecting them into human cancer cells. The development of such cell lines currently is ongoing in our lab. Furthermore, *in vivo* studies using the human Met, either in mouse cells or human epithelial cells with low endogenous Met expression, will be free from HGF stimulation as murine HGF is unable to activate human Met[206, 299]. Therefore, grafting these cells

into nude mice would allow us to more clearly assess the intrinsic properties of these mutations.

A study by Cho et al. was published at the end of my PhD suggesting a possible dileucine motif in the C-terminus of Met (amino acids 1378 to 1393) that is involved in its endocytosis through binding to adaptin β1/2 and caveolin[300]. They developed a specific peptide that competed with Met for the binding to the endocytic machinery[300]. However, we have shown that M1268T Met, as well as Wt Met stimulated with HGF[62, 159], appears to internalise via different mechanisms i.e. using dynamin, clathrin, Grb2 and c-Cbl. Furthermore, in this paper, it was not clearly demonstrated that Met internalisation was blocked and thus moreover was perhaps not specific to Met as it was compared to Glut4 and Transferrin receptors but to other RTKs. Therefore, although it forms an interesting idea that a peptide may be developed as a potential treatment, further work is required to investigate whether it could reduce the oncogenicty of Met mutants specifically.

Endosomal signalling is required for Met mutant expressing cells' migration and tumourigenesis

Using our various methods to block Met mutants' endocytosis, we observed that the internalisation of Met mutants is required for the transforming capacity *in vitro* and *in vivo*. Here I have shown that Met mutants M1268T and D1246N internalisation through Grb2 and c-Cbl dependent mechanisms is required for their stimulation of cell migration. As already discussed in Chapter 1, there are relatively few studies that demonstrate a role for

endosomal signalling in cell migration. Palamedessi et al. however recently demonstrated that Rac is activated by its GEF Tiam1 on endosomes upon HGF stimulation or Rab5 expression[158]. This is of particular interest to us, as the migration of cells expressing M1268T Met is dependent on Rac1 activation and a requirement for the localisation of M1268T Met on endosomes. Thus perhaps in our model M1268T Met activates Rac on endosomes through the recruitment of Tiam1 and this would certainly be interesting to investigate.

Importantly, I showed that blocking endocytosis, through either abolition of clathrin, Grb2 or c-Cbl expression, has no effect on Met mutants M1268T and D1246N activation *in vitro* but does reduce their ability to undergo anchorage independent growth. Furthermore, abolition of clathrin expression *in vivo* has no effect on Met M1268T activation but does bring about a significant size reduction of M1268T tumours (**Figures 16**). Together with the fact that Met mutants' oncogenicity can be reduced to a similar level using Met inhibition, the reduction of tumourigenicity that occurs following endocytosis inhibition appears to be a consequence of the Met localisation in addition to its activation status. Thus, it is the constitutive internalisation together with the high activation status that makes the Met mutant highly tumourigenic.

This shows that, despite the traditional view that it is RTK activation alone that drives tumourigenesis, in fact the localisation of this constitutively active

RTK on endosomes is absolutely necessary for it to fulfil its full oncogenic potential.

Endosomal signalling of RTKs and Cancer

It is recognised that RTKs can induce tumourigenesis through overexpression, activating mutations or excessive paracrine/autocrine signaling and normally the cellular location of an RTK is not investigated. Thus it has previously been thought that the high activation status of the D1246N and M1268T mutants causes them to drive tumourigenesis [286, 294]. Nevertheless, a few studies have described constitutively active RTKs, that signal from intracellular compartments. These include KIT D814Y, which was found signalling from the Golgi[301] as well as RET R918T, FGFR3 and Flt3-ITD which were all found signalling from the endoplasmic reticulum[302-304]. However they were all signalling in their immature forms as they trafficked through the secretory pathway, but nevertheless the signalling from these locations was found to be able to promote tumourigenesis[301, 305].

Our observations, instead point towards “endosomal signalling” of a mature RTK following trafficking from the plasma membrane[306-308]. This form of signalling has been suggested to provide a way of enhancing signal specificity, which would seem to be especially important for the Met RTK, which transduces all of its downstream signalling from the Y1349 and Y1356 tyrosines in the Met docking site[309]. Furthermore, endosomal signalling of Met has been shown to induce this specificity[62, 157, 310].

3) <u>Met Oncogenic Mutants: Sensitivity to HSP90 inhibitors</u>

Reduced degradation of RTKs has been shown to occur in cancer[311]. Here I have confirmed that both the M1268T and D1246N Met mutants suffer from a defect in degradation as compared to Wt Met as previously described by Dr. Carine Joffre.

Met mutant degradation can be restored through inhibition of HSP90, resulting in reduction in cell migration and tumourigenesis

Previously, Met mutations that induce a defect in degradation have been identified in lung cancer[201]. These mutations are somatic intronic deletions that lead to an alternatively spliced variant of Met lacking the juxtamembrane domain, and consequently the Y1003 c-Cbl binding site[201]. When a Y1003F Met mutant was generated that could not bind c-Cbl, Met still underwent endocytosis and trafficking with similar kinetics to WT Met, however it was not able to be degraded[201]. However, our study is the first to report a defect in degradation of Met mutated in the kinase domain. Moreover, so far no Met mutants that have a defect in Met degradation and an increased Met internalisation have been described.

Unpublished data from Dr. Carine Joffre have demonstrated that the M1268T mutant does not have a defect in Y1003 phosphorylation or Met ubiquitination and in fact if anything M1268T Met is more ubiquitinated than the Wt Met. Thus, the defect in degradation of the mutants observed is not due to a lack of ubiquitination. However, I showed that HSP90 activity is responsible for the mutants' stability, consistent with Carine Joffre finding that

the Met mutant M1268T is constitutively associated with HSP90. This importantly suggests that HSP90 protects Met from degradation directly rather than via another protein. HSP90 is a molecular chaperone that is involved in the stabilisation of various proteins in their correct conformation and it has also been shown to be involved in protecting Wt Met from degradation[91]. In our study we have used the HSP90 inhibitor 17-AAG, which is a derivative of geldanamycins. In fact, geldanamycins have been shown to induce downregulation of the basal expression of the mutated Met L1213V/M1268T and revert the malignant associated phenotype of cells carrying this mutation[134]. This is the only study published so far that has investigated the effect of HSP90 inhibition on a Met mutation, however it did not notice a *defect* in Met degradation and did not investigate the effects on the Met induced tumourigenicity.

I have shown that inhibition or knock down of HSP90 restores the degradation of the Met mutants M1268T and D1246N, both upon HGF and independently of HGF (**Figure 17)**. Furthermore, I have shown that inhibition of HSP90 in the M1268T cells led to a reduction in anchorage independent growth and cell migration *in vitro* as well as a reduction of tumourigenesis *in vivo* (**Figure 18AB and 20BC**). Meanwhile, *in vitro*, there was no or very little effect on Wt Met expressing cells (which are not transformed). This, together with the fact that the transformation of the mutant cells is dependent on Met activity, suggests that this approach appears to impair Met mutants' dependent cell transformation specifically.

D1246N mutant requires a higher dose of 17-AAG for efficient inhibition of Met dependent tumourigenesis

Although inhibition of HSP90 also restored the degradation of the D1246N mutant, the same dose of 17-AAG did not seem to be as effective at inhibiting the anchorage independent growth of the cells expressing this mutant (24.4% decrease) compared to the M1268T cells (greater than 46% decrease). However, depletion of HSP90 using RNAi achieved a larger decrease in anchorage independent growth in the D1246N Met cells, similar to that obtained in the M1268T cells. While in M1268T Met expressing cells 100nM 17-AAG and HSP90 RNAi reduced the anchorage independent growth to a similar level. This suggests that 17-AAG is not, over the time of the soft agar assays, as effective at restoring D1246N Met degradation through HSP90 inhibition as it is for M1268T Met. Consistent with this, the rate of D1246N Met degradation upon 4 hours of HGF stimulation following HSP90 inhibition appeared to be slower than that of the M1268T Met **(Figure 18B**). The reason for the difference in sensitivity to 17-AAG between the two Met mutants could also be due to the conformational differences previously discussed[294] between their mutations (Discussion Chapter II-1), which perhaps alter the way that they bind to HSP90 or to the inhibitors (Met TKI and 17-AAG).

A dose dependent response to 17-AAG was however observed in D1246N Met degradation and the mutant expressing cells' anchorage independent growth. Thus 200nM 17-AAG significantly decreased their anchorage independent growth to a similar level as following transfection with HSP90

RNAi, suggesting that specificity is still maintained at this dose. The IC50 of 17-AAG varies depending on the cell type and especially whether the cells are normal or tumourigenic. For example, the IC50 of various cancer cell lines averages at 39nM. However, the IC50 is lower in Her2-positive breast cancer cell lines (oncogene driven), being 5nM, and much higher in normal primary cells, being 943nM[124]. Thus the dose of 200nM still remains within the range at which it should not affect normal cells at this dose.

HSP90 Inhibition as cancer treatment for Met dependent cancers in the clinic

HSP90 inhibitors are currently in phase I, II as well as a few phase III clinical trials for the treatment of various cancers (http://www.clinicaltrials.gov). Wang et al. showed that treatment of cancer cells harbouring Met amplification with 17-AAG led to a durable decrease in the activation of Met and its downstream signalling molecules as well as inhibition of cell growth[135]. Furthermore, these effects were greater and more durable than those observed using the Met inhibitor SU11274[135]. These studies, together with the effective inhibition of Met M1268T dependent tumour growth that we observed in M1268T tumours using 17-AAG, suggest that treatment of patients bearing the M1268T mutation with HSP90 inhibitors may provide a very effective long-term therapy.

This project is still ongoing and the next step will be to attempt treating D1246N tumours *in vivo* with 200nM 17-AAG. The *in vitro* data gathered so far provide promising results and suggest that 17-AAG may be effective at

reducing the growth of D1246N expressing tumours *in vivo*. If so, it will provide proof of principle that 17-AAG can be used to treat Met driven cancers, even in situations where Met is resistant to TKIs.

One advantage of using HSP90 inhibitors where effective is that they have multiple indirect but cancer driving targets, as HSP90 has a very wide range of cancer-associated clients. They may therefore produce a more effective or durable response than Met TKIs. It is important to note however that acquired resistance to HSP90 inhibitors has been documented experimentally[312]. The current investigations on the use of HSP90 inhibitors in long-term cancer treatment will inform further on potential resistance to such inhibitors, which, otherwise appear very promising.

HSP90 α expression is increased in M1268T expressing cells, however both isoforms α and β appear to play a role in cell functions

In this study I have found that the M1268T Met oncogenic mutant expressing cells have increased expression of the HSP90 α isoform compared to Wt expressing cells. This is perhaps not surprising as the HSP90 α isoform has previously been shown to be upregulated in various tumours[94].

Investigation into the functional role of each HSP90 isoform in the M1268T expressing cells revealed that in fact either HSP90 α or HSP90 β isoform is required for M1268T Met dependent cell migration. Intriguingly, however, knockdown of both isoforms together significantly increased the migration of the Wt Met expressing cells, which correlated with increased Wt cell

migration observed following silencing of the HSP90 β isoform alone. Thus, although targeting either isoform can inhibit cell migration, possibly targeting the HSP90 α isoform alone has less effect on non-tumourigenic cells. This may be due to compensation by the HSP90 α isoform in the absence of HSP90 β, such that inhibition of HSP90 β knockdown may increase the expression of HSP90 α, which has a currently unknown role to play in general cell migration. This could be potentially interesting to investigate given the increased expression of HSP90 α in tumourigenic cells[94], including the M1268T mutant.

In a contrasting manner, only the depletion of both isoforms together had an effect on the anchorage independent growth of the M1268T expressing cells. However, this result could be due to difference in the time-span of the experiments, as soft agar assays last 6 days, at which point expression levels of the silenced proteins may have partially returned and perhaps when both proteins were silenced simultaneously the threshold of knockdown required to inhibit the cell growth in soft agar over 6 days might be achieved. In contrast, Transwell migration assays are performed 72 hours after transfection at the point of optimum protein depletion and last only 4 hours. Thus, another more comparable way to assess anchorage independent growth needs to be carried out. This could possibly be achieved by fluorescently labelling the cells, allowing the experiment to be stopped earlier (e.g. day 3) and then analysing by the more sensitive method of measuring fluorescence intensity produced by the colonies formed.

Inhibition of co-chaperone-HSP90 interactions

Inhibition of the interaction between HSP90 and various co-chaperones has been proposed to provide an alternative way to target HSP90 client proteins more specifically. We have hypothesised that Hop may be involved in the stability of Met mutants. However, I generated inconclusive experiments with opposite results. The reasons for these discrepancies may be due to difference in knockdown efficiency. In the first experiment, Hop silencing led to a decrease in Met expression, which was not surprising as it is known that Hop is involved in delivering client proteins to HSP90. Thus Hop silencing is expected to lead to Met degradation. However, in the second experiment, I observed an increase in Met expression. As in this experiment, Hop silencing was more efficient; perhaps there is some compensation between other members of the HSP90 complex. Certainly further repetitions are required to establish what constitutes the real result.

Another co-chaperone, CHIP, would be particularly interesting to investigate in M1268T expressing cells, as it has been found to associate with the constitutively active M1254T Ron mutant and is responsible for Ron degradation upon 17-AAG treatment[130]. This mutant is highly sensitive to 17-AAG treatment and it could, therefore, be interesting to investigate whether there is a difference in the association between CHIP and the two Met oncogenic mutants studied here, as perhaps there is an increased association between CHIP and M1268T which could explain its increased sensitivity to HSP90 inhibition over the D1246N mutant.

Interestingly, Novobiacin, a coumarin antibiotic, binds to HSP90 in its C-terminal domain and alters its interactions with co-chaperones[94, 117]. While Novobiocin itself has a low affinity for HSP90, several derivatives have been produced that have a higher affinity for HSP90 than 17-AAG and appear to have good anti-proliferative effects on several cancer cell lines[93]. Thus, it would be interesting to test this inhibitor on the D1246N and M1268T Met mutants to see if it is more effective than 17-AAG and if it alters interactions between Met, HSP90 and, as yet, unknown co-chaperones.

Degradation versus Endocytosis

One of the unanswered questions is whether the mutants recycle due to their defect in degradation? Or do they not degrade due to their increased recycling? Unpublished data from our lab have demonstrated that the Met mutants are not detected on late endosomes or on lysosomes. Thus, firstly, having restored Met degradation, it would be interesting to investigate if the trafficking of the Met mutants is restored to the late endosomes. Secondly, it would be interesting to block the proteosome/lysosomal degradation of both Wt cells and M1268T cells treated with 17-AAG and see if Met accumulates in late endosomes or if recycling is increased. Finally, it would also be interesting to do the inverse experiment and see if the impairment of Met mutants' recycling restores their degradation.

Interestingly, inhibition of M1268T tumourigenesis *in vivo* was inhibited to an equal degree by both 17-AAG and Dynasore, suggesting that inhibition of the accumulation of M1268T Met on endosomes, either by inhibition of

endocytosis or by restoration of Met degradation, is equally sufficient to reduce tumour growth.

Many studies have reported RTKs that avoid degradation[88, 193, 288, 313], and others have reported increased recycling of RTKs in cancer[185, 187, 189, 194]. However, these studies tend to assume that the resulting mechanism for increased tumourigenicity, is an increased life-span of the receptor in question. They do not question whether the location of the receptor is important. Whereas we have demonstrated that it indeed is the intracellular location of Met that is required for tumourigenesis.

Results Part III: β1-integrin - Met Crosstalk: an endosomal inside-in signalling

1) β1-integrin co-traffics with Met and is required for Met dependent cell migration and ERK 1/2 phosphorylation

Crosstalk between various different integrins and RTKs has been shown to occur. In this study, I have investigated the relationship between Met and β1-integrin, the ubiquitously expressed β1-subunit of most ECM binding integrins, including α5β1, the major receptor for fibronectin[232, 248, 314]. To investigate the crosstalk between these two major cell surface receptors, I used β1-integrin null GD25 cells generated from early β1-integrin null mouse embryos[315] ("GD25 cells") and the same cells re-expressing β1-integrin ("β1A cells") (**Figure 1A**)[263].

I firstly wanted to investigate the role of β1-integrin in Met dependent cell migration. Interestingly, in scratched confluent monolayers, HGF increased the wound healing of β1A cells significantly (by more than 2 fold, $p<0.01$) but had no effect in GD25 cells (**Figure 1BC**). Dr Stephanie Kermorgant showed that PDGF, however, was able to increase the wound healing of GD25 cells (**Appendix Figure 5A**), showing that these cells are inherently able to migrate.

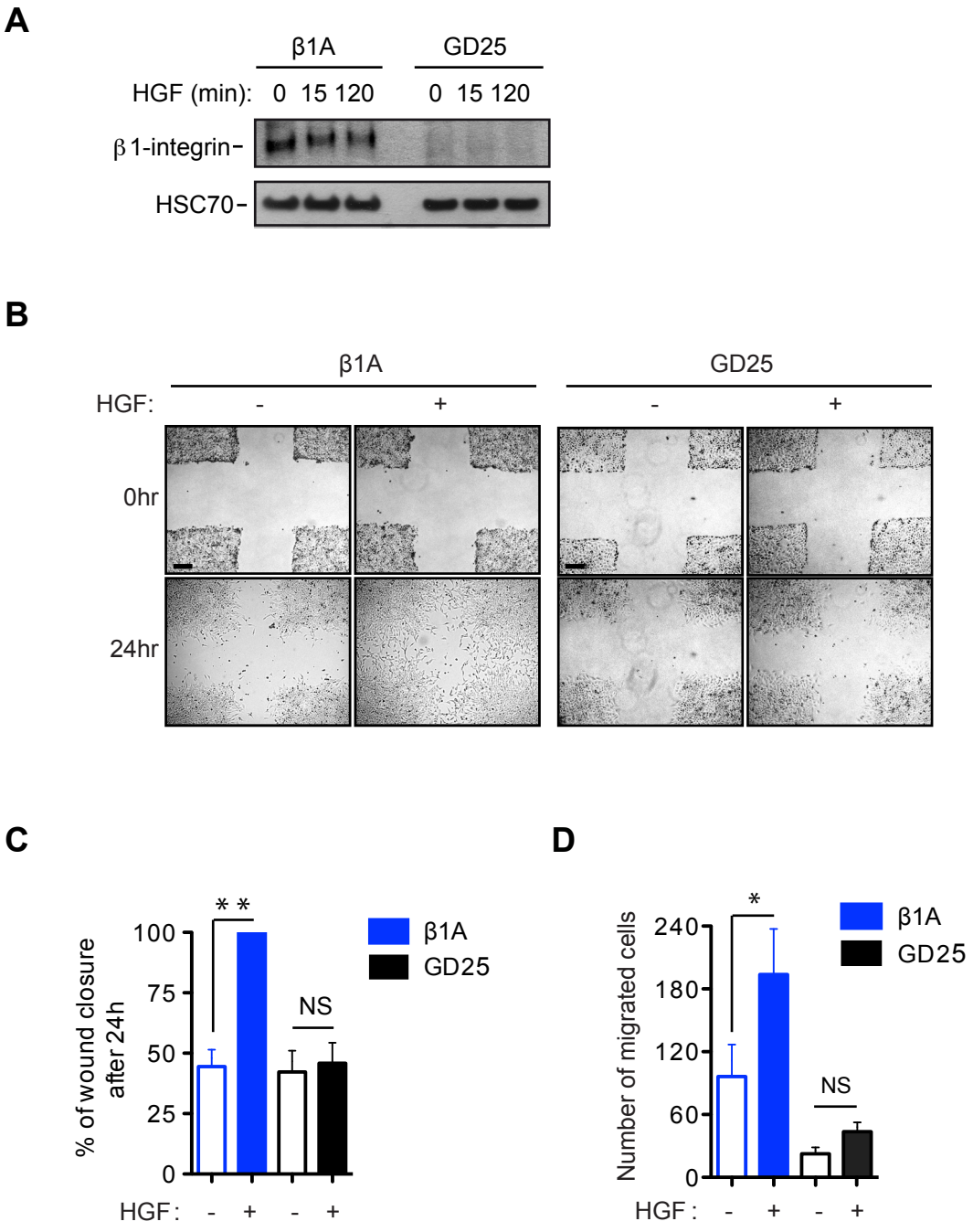


Figure 1: β1-integrin is required for Met dependent cell migration
A) Western blots for β1-integrin, HSC70 in β1A, GD25 and β1A-YYFF cells stimulated with HGF for 0, 15 and 120 minutes. **B)** Pictures of confluent β1A or GD25 cells just after wounding (0) or 24 hours after wounding. Cells were treated or not with HGF. Scale bar: 130μm. **C)** Quantitation of wound closure. Data are mean (arbitrary units) ± SEM (n=5). **D)** Average number of the indicated cells that have migrated through Transwells over 2 hours of incubation with HGF. Data are mean ± SEM (n=3), each experiment done in triplicate. *p<0.05; **p<0.01.

In order to confirm my observations in the wound healing assays, I used Transwell migration assays to investigate the Met dependent migration of these cells further. HGF significantly increased the number of β1A cells that migrated by more than 2 fold ($p<0.05$), while no significant increase in migration occurred in GD25 cells (**Figure 1D**). These results indicated that β1-integrin is required for HGF/Met dependent cell migration. Furthermore, β1-integrin could not be compensated for by fibronectin-binding β3-integrin heterodimers, as they are expressed in both of these cell types[263].

Since β1 trafficking (cycles of endocytosis and recycling) recently has been shown to play a substantial role in cell migration[231, 248, 316, 317], I investigated whether the HGF / Met pathway influences the trafficking of β1-integrin.

Flow cytometry experiments carried out in the laboratory of our collaborator Prof. Johanna Ivaska showed that cell surface expression of β1-integrin was reduced rapidly at the plasma membrane following short periods of HGF stimulation in β1A cells (**Appendix Figure 5B**), indicating integrin internalisation. They confirmed this through performing a biotin-based internalisation assay, where, upon 15 minutes of incubation, a higher proportion of surface biotinylated β1-integrin was internalised in the presence of HGF relative to in the absence of HGF (**Appendix Figure 5C**). I confirm these findings by immunofluorescence. Analysis by confocal microscopy showed that, at 30 minutes of HGF stimulation, there was an increased amount of β1-integrin in the intracellular pool compared to no stimulation (**Figure 2A**). I have used the fluorescently labelled HGF (HGF*) to follow the

localisation of HGF-bound Met[13]. As expected, punctate HGF* staining was observed at 30 minutes, indicating the internalisation of Met upon HGF stimulation[82], which also was seen in GD25 cells **(Figure 2B)** showing that β1-integrin is not required for Met to be able to undergo endocytosis. Strikingly, colocalisations between β1−integrin and HGF* were observed in intracellular vesicles, indicating Met/HGF and β1-integrin co-traffic and suggesting that they might co-internalise (**Figure 2A**).

Due to the fact that HGF dependent cell migration requires Met signalling from endosomes[62, 157, 159], I tested whether β1-integrin could have a role in Met endosomal signalling. In β1A cells, HGF triggered a robust (x 5 fold increase at 15 minutes, $p<0.01$) and sustained (x 2.6 fold increase at 120 minutes, $p<0.05$) ERK1/2 phosphorylation. Surprisingly, by contrast, in cells lacking β1-integrin (GD25), the intensity of the ERK1/2 signal was reduced by over 40 % at 15 minutes ($p<0.05$), and was not sustained over time (NS) (**Figure 3A)**. However, Met expression levels **(Figure 3B)** and, moreover, Met phosphorylation were not affected (**Figure 3B**), indicating β1-integrin may influence downstream signalling of Met rather than affecting Met directly. Taken together, these results suggest that β1-integrin influences the Met-dependent ERK1/2 pathway on endosomes.

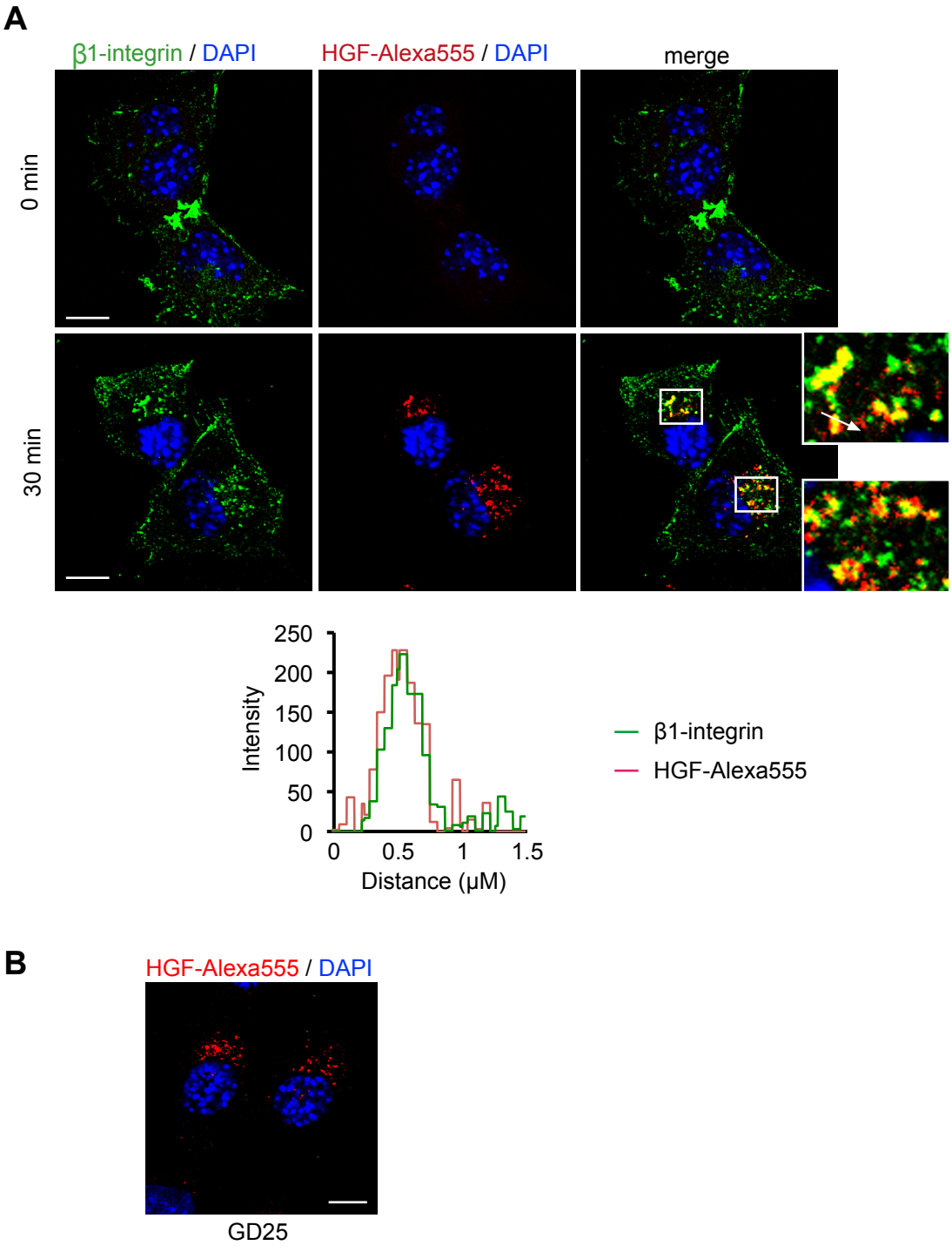


Figure 2: β1-integrin co-traffics with Met

A) Confocal sections of β1A cells, stimulated, or not, with HGF-Alexa555 (red) for 30 minutes, and stained for β1-integrin (green) and DAPI (blue). Co-localisations appear in yellow. Scale bars: 10 μm. Plots of the intensity profiles, at different emission wavelengths corresponding to the signals of β1-integrin and HGF-Alexa555, of a set of pixels distributed on a line drawn across a vesicle (shown in the top panel magnification) are shown.

B) Confocal section of GD25 cells stimulated with HGF-Alexa555 (red) and stained for DAPI (blue). Scale bar: 10μm.

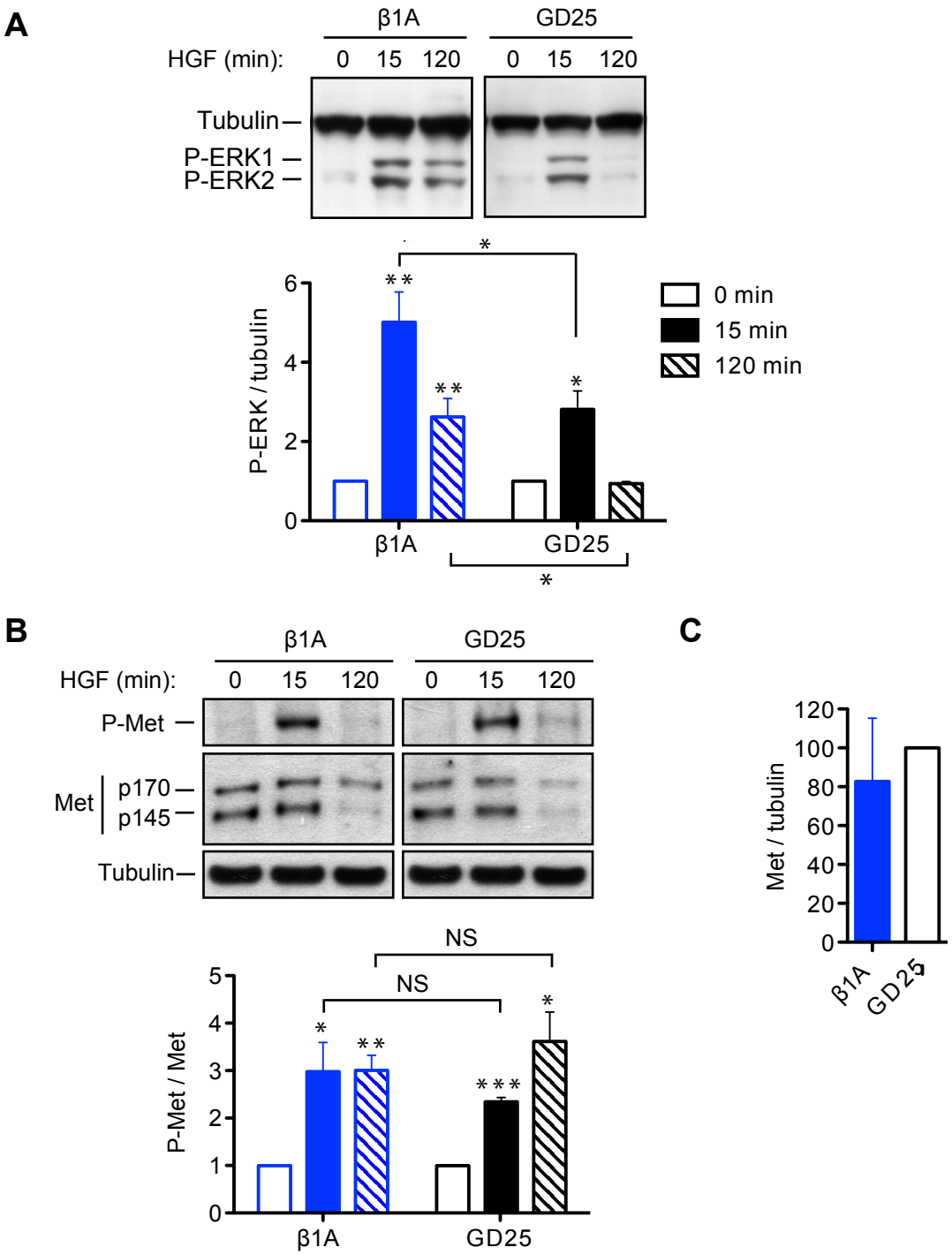


Figure 3: β1-integrin plays a role in Met dependent ERK1/2 phosphorylation

A) Western blots for tubulin and phospho-ERK1/2 in β1A and GD25 cells, stimulated with HGF for 0, 15 and 120 minutes. Numbers represent fold increases ± SEM (n=6) of phospho-ERK1/2 / tubulin ratios at 15 and 120 minutes over 0 minutes. **B)** Western blots for phospho-Met (Y1234/35), total Met (p170, precursor, p145: mature β chain) and tubulin. Numbers represent mean ± SEM (arbitrary units, n=4) of phospho-Met / Met p-145 ratios.
C) Quantitation of Western Blots. Numbers represent relative levels (arbitrary data) ± SEM (n=3) of Met / tubulin ratios in β1A versus GD25 cells. *p<0.05; **p<0.01; ***p<0.001; NS=not significant.

The cytoplasmic domains of β-integrins contain two conserved NXXY motifs, which have been implicated in integrin function. Both motifs have been shown to regulate integrin activation[231], to play a role in the signalling of β3-integrin to Shc[318] and to be critical for β1-integrin mediated activation of FAK[319] . I therefore analysed the influence of the double mutation, Y783F/Y795F, in the NXXY motifs of β1-integrin. In cells expressing the β1-integrin "YYFF mutant" ("β1A-YYFF cells"), ERK1/2 activation upon HGF stimulation was similar to that observed in GD25 cells (fold increases were not significantly different at 15 and 120 minutes) (**Figure 4A**). Although ERK1/2 phosphorylation was stimulated at 15 minutes ($p<0.05$), the intensity was reduced by nearly 50% compared to β1A cells at the same time ($p<0.05$). Furthermore, correlating with what was found in GD25 cells, the ERK1/2 signal was not sustained (**Figure 4A**)**.** Meanwhile, Met phosphorylation and expression levels were unchanged (**Figure 4B**). The reduction in HGF-dependent ERK1/2 phosphorylation was not the result of a decrease in total ERK1/2 expression levels in GD25/β1A-YYFF cells compared to β1A, which were checked by Western blot (**Figure 4C**). The kinetics of Met degradation in all three cell lines were also unchanged (**Figure 4D**). Interestingly, in wound healing assays β1A-YYFF cells did not respond to HGF significantly (**Figure 5A**) thereby resembling GD25 cells (**Figure 1BC**). It is also important to note that the lack of β1-integrin (GD25 cells) or the presence of β1A-YYFF in cells does not seem to alter the growth rate of the cell lines, such that in fact the GD25 cells grow slightly faster (non

significant) than the β1A and β1A-YYFF cells (**Figure 5B**).

Thus, it can be concluded that the participation of β1-integrin in Met endosomal signalling, required to stimulate cell migration, is dependent on a signaling competent β1-integrin with intact cytoplasmic NXXY motifs.

2) β1-integrin co-traffics with Met and is required for Met ERK1/2 phosphorylation in cells existing in suspension

All the results so far were obtained with cells cultured directly on plastic. Dr. Stephanie Kermorgant has performed some experiments to assess the influence of β1-integrin ligands on β1-integrin-Met dependent ERK1/2 activation. β1A cells were trypsinised, the trypsin was neutralised with soybean trypsin inhibitor and cells plated on laminin or fibronectin or on the control substrate poly-L-lysine for different periods of time with or without HGF. Surprisingly, HGF activated ERK1/2 to a similar extent under each condition (**Appendix 5D**). This result suggested that the β1-integrin input in Met dependent ERK1/2 activation was unrelated to β1 substrate engagement and also that β3-integrin plays no role in this process, since fibronectin is also a ligand for β3-integrin whereas laminin binds specifically only β1-integrins. To assess further whether Met dependent ERK1/2 activation was independent of β1 ligand, β1A cells, serum-starved for 24h, were trypsinised, the trypsin was neutralised with soybean trypsin inhibitor and cells were cultured in serum-free medium under non-adherent conditions for 1 hour followed by HGF stimulation for 15 and 120 minutes. At 15 minutes, HGF

was able to stimulate ERK1/2 phosphorylation to a level similar to that in adherent cells, confirming integrin ligand- and anchorage- independence (**Appendix Figure 5E**). Interestingly the ERK1/2 activation in these suspension cultures was more sustained than in adherent cultures (**Appendix Figure 5E**).

These results on cells in suspension prompted us to extend this study to a different cell system that Dr Stephanie Kermorgant had previously generated, as a post-doc in Prof. Peter Parker's laboratory, where cells lose adherence and grow in suspension in response to Met activity. This cell system, "Met-GFP cells", consists of HEK-293 cells with tetracycline-inducible Met-GFP expression (TET ON system). Met-GFP expression was detected after 3 hours of incubation with 0.1 μg/ml tetracycline and the level increased with the time of induction (**Figure 6A**). Here I present experiments that I have performed (unless otherwise stated), however for the majority of them, it was a repeat of experiments done by others in order to reach at least n=3.

Interestingly, when expressed, Met-GFP was phosphorylated constitutively (**Figure 6A**) (most likely due to GFP-GFP oligomerisation and subsequent trans-phosphorylation of Met-GFP molecules), therefore also providing us with a model to investigate Met activity, which in this case occurs independently of HGF. Furthermore, cell rounding and detachment from the plate occurred, which temporally coincided with the induction of Met-GFP. The detached cells formed floating spherical colonies (**Figure 6B)** with retained Met-GFP activity after 16 hours (**Figure 6C**). Flow cytometry analysis (performed by Nick Luckens and Dr. Stephanie Kermorgant), for

propidium iodide staining of Met-GFP cells which were non-induced and induced for 48 hours with 0.1 μg/ml of tetracycline, showed that the Met-GFP expressing cells remained viable despite their detachment from the plastic (**Appendix Figure 6A**). Moreover, the Met-GFP expression, and consequent cellular phenotype, was reversible with tetracycline removal indicating that the loss of adhesion, occurring with Met-GFP expression, did not induce anoikis. Importantly, cells re-attached rapidly upon addition of the Met specific inhibitor SU11274 for just 1 hour (**Figure 6B**), while they did not upon addition of the unrelated VEGFR2 inhibitor SU1498. Thus cell detachment is dependent on Met activity.

ERK1/2 was activated progressively following tetracycline induction (**Figure 6A**), remaining activated after 16 hours when cells were fully detached (**Figure 6C**). Treatment with SU11274 inhibited ERK1/2 phosphorylation, indicating that ERK1/2 activation is the result of sustained Met action (**Figure 6C**).

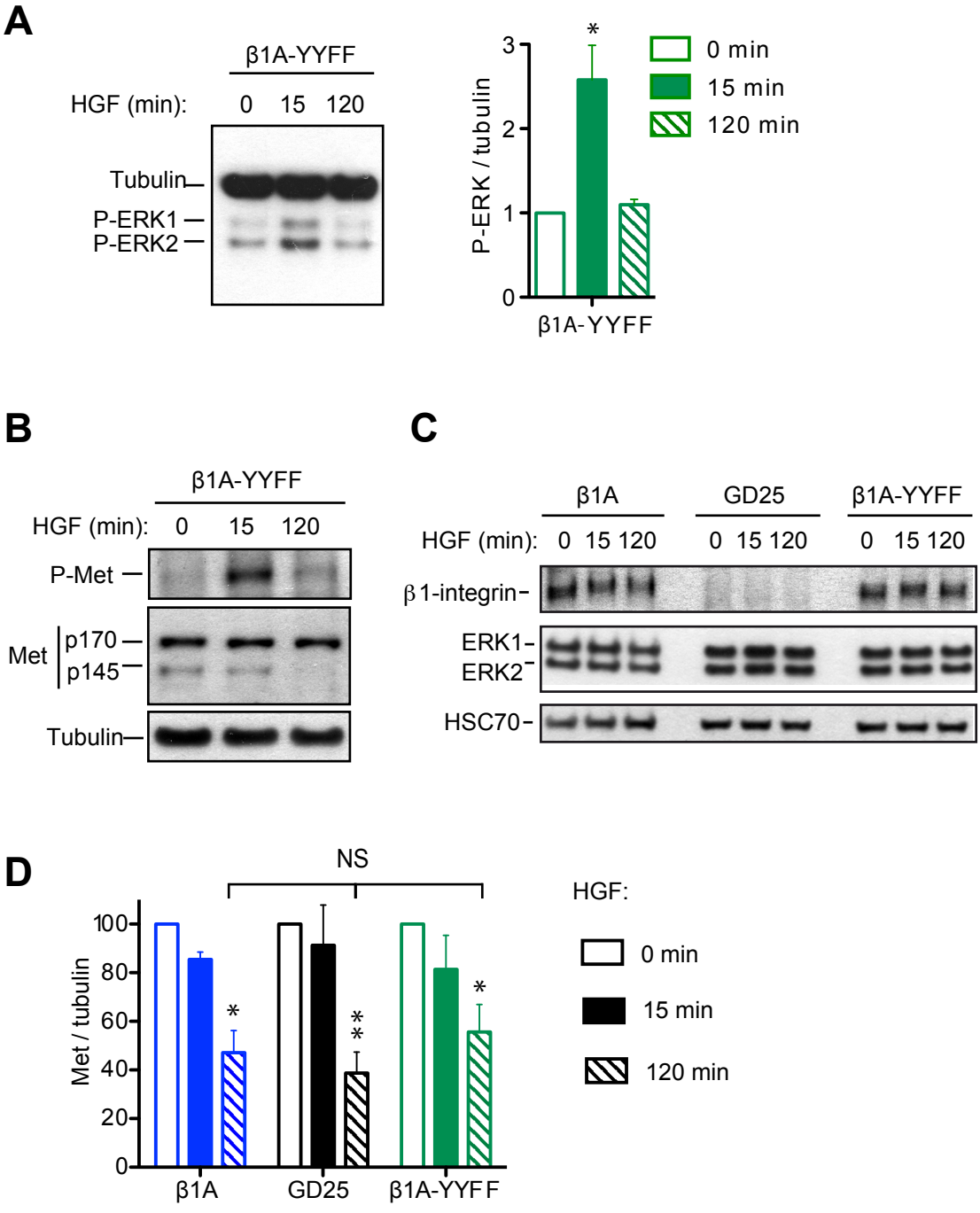


Figure 4: The conserved YYFF motif of β1-integrin plays a role in Met dependent ERK1/2 phosphorylation

A) Western blots for tubulin and P-ERK1/2 in β1A-YYFF cells, stimulated with HGF for 0, 15 and 120 minutes. Numbers represent fold increases ± SEM (n=6) of P-ERK1/2 / tubulin ratios at 15 and 120 min over 0 minutes. **B)** Western blots for phospho-Met (Y1234/35), total Met and tubulin in YYFF cells, stimulated with HGF for 0, 15 and 120 min. **C)** Western blots for β1-integrin, pan-ERK 1/2 and HSC70 in β1A, GD25 and β1A-YYFF cells stimulated with HGF for 0, 15 and 120 minutes. **D)** Quantitation of Western blots. Numbers represent levels of Met protein (p145-Met / tubulin ratios) upon time of HGF stimulation (arbitrary data) ± SEM (n=3). *p<0.05; **p<0.01; ***p<0.001; NS=not significant.

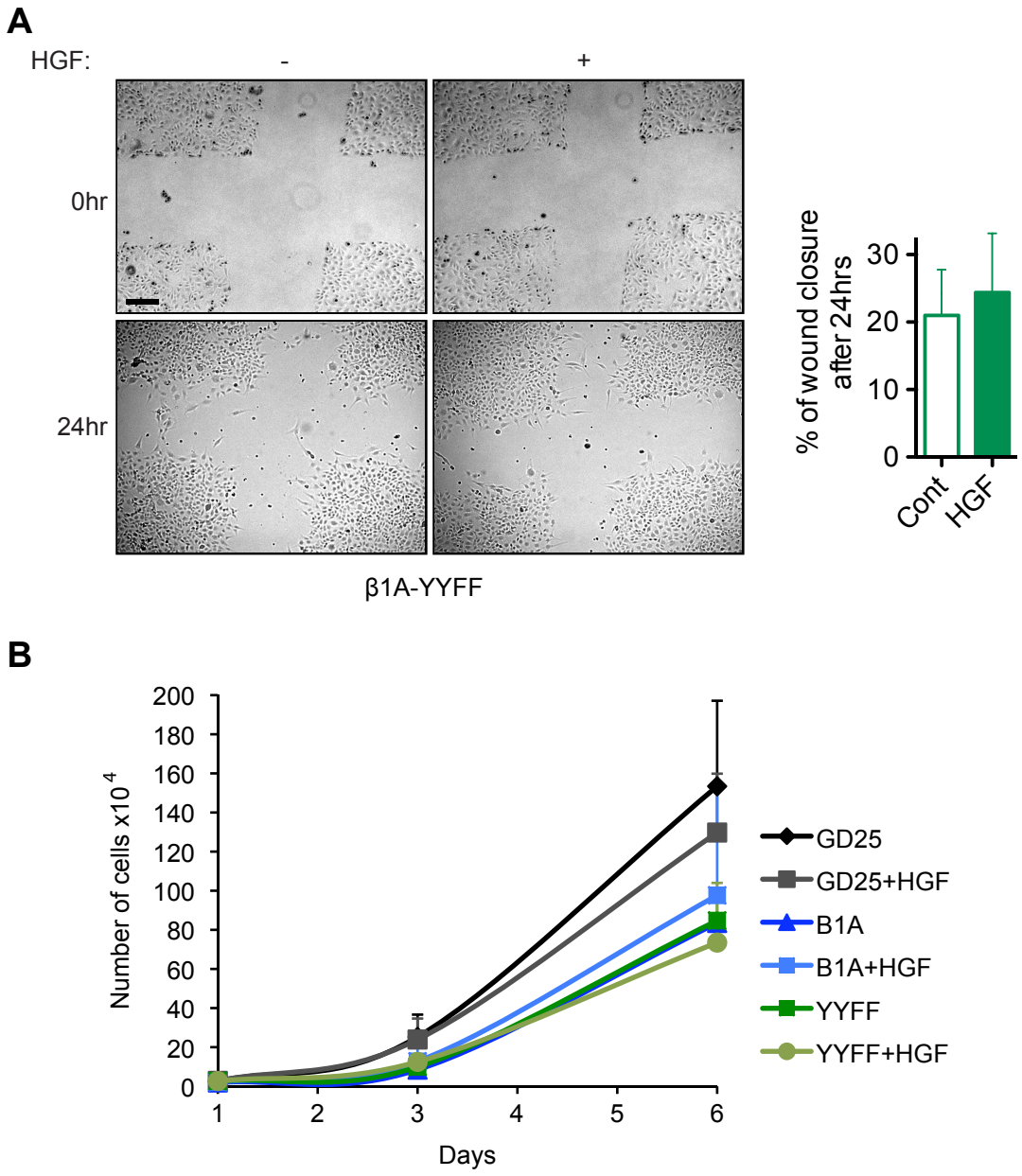


Figure 5: The YYFF motif of β1-integrin plays a role in Met dependent cell migration

A) Pictures of confluent β1A-YYFF cells just after wounding (0 hours) or 24 hours after wounding. Cells were treated or not with HGF. Bar: 130µm. Graph shows the quantitation of wound closure for β1A-YYFF cells. Data are mean (arbitrary units) ± SEM (n=3). *p<0.05; **p<0.01; ***p<0.001.
B) Growth curve of GD25, β1A and β1A-YYFF cells incubated +/- HGF. Cells were counted on Day 3 and Day 6. Data are mean (arbitrary units) ± SEM (n=2).

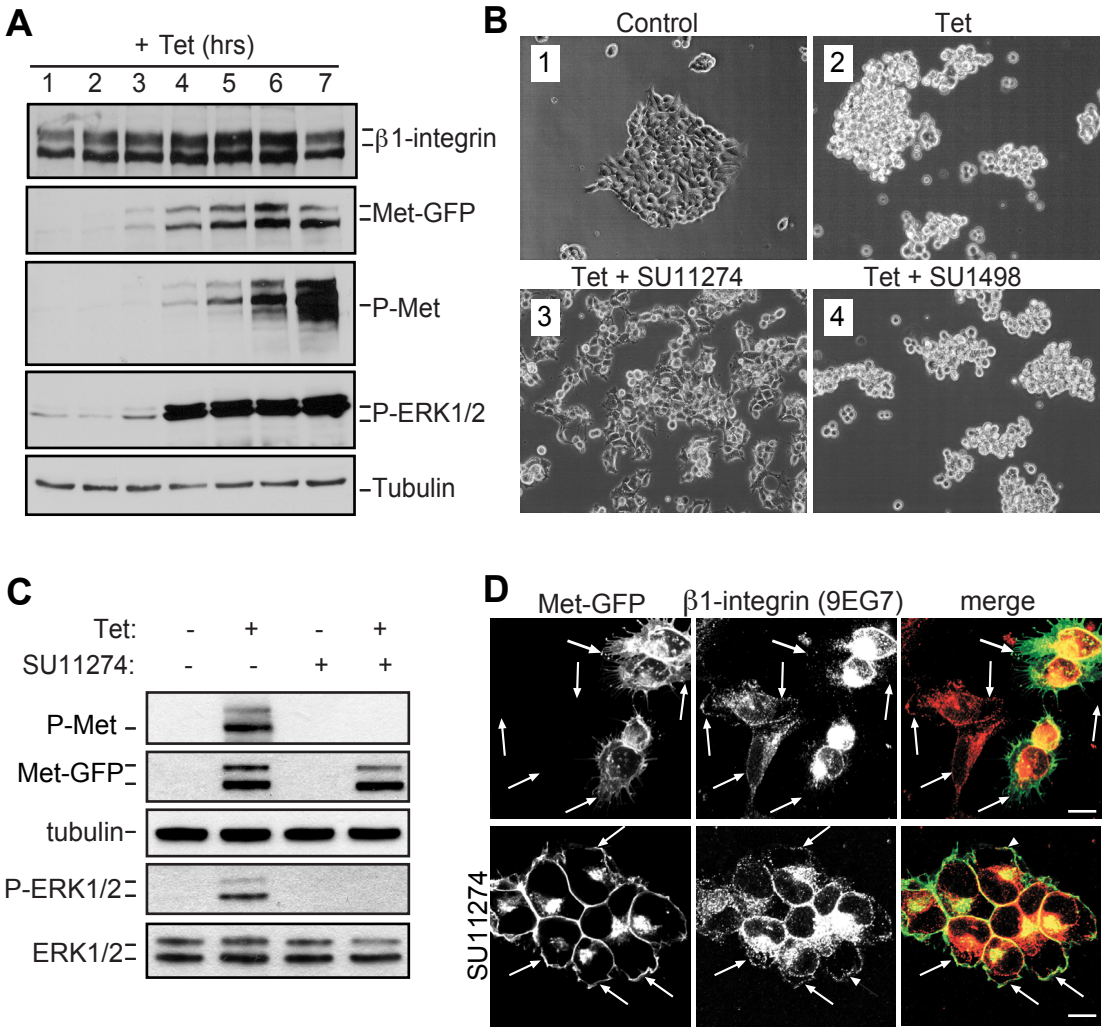


Figure 6: β1-integrin co-traffics with activated Met-GFP and plays a role in Met-GFP signalling to ERK1/2 in cells growing in suspension
A) Western blots for β1-integrin, GFP (Met-GFP: p195, precursor; p170, mature β chain), phospho-Met (Y1234/35), phospho-ERK1/2 and tubulin in Met-GFP cells, incubated in tetracycline (Tet, 0.1µg/ml) for the times indicated in hours (hrs). **B)** Low light images of Met-GFP cells, incubated or not (control) in tetracycline (Tet, 0.1 µg/ml) for 16 hours, further incubated for 1 hour or not in the Met inhibitor SU11274 or the unrelated VEGFR2 inhibitor SU1498. Image 1: cells are attached; Images 2 and 4: cells have detached; Image 3: cells have re-attached. **C)** Western blots for phospho-Met (Y1234/35), GFP (Met-GFP: p195, precursor; p170, mature β chain), P-ERK1/2, pan-ERK1/2 and tubulin in Met-GFP cells, incubated in tetracycline (Tet, 0.1µg/ml) for 0 or 16 hours and treated for 1 hour or not with the Met inhibitor SU11274. **D)** Confocal projections of 7 Z-sections from the base to the apex of the cell. Arrows show examples of plasma membrane staining. Scale bar, 10µm. Cells were cultured on Poly-L-lysine coated glass coverslips for 16 hours in the presence of 0.1 µg/ml tetracycline. Cells were stained for anti-β1-integrin in active conformation (9EG7) (red). Met-GFP is in green. Top panel: T-REx™-293 cells, non-transfected and stably transfected with Met-GFP ("Met-GFP cells"), at a 50/50 ratio. Bottom panel: T-REx™-293 cells stably transfected with Met-GFP ("Met-GFP cells") and treated with SU11274 (2µM).

We found that Met-GFP-induced cell rounding coincided with a decrease of β1-integrin from the cell surface. Flow cytometry analysis of pan β1-integrin expression on the cell surface, performed in Dr. Ivaska's laboratory, demonstrated that the reduction reached 60% at 3 hours ($p<0.05$) (**Appendix Figure 6B**), whereas the total cellular β1 levels remained unaltered (**Figure 6A**). Concomitantly, β1 internalisation was increased, as measured by a biotinylation-based endocytosis assay, over 1-5 hours of tetracycline induction ($p<0.001$ at 2 hours) (**Appendix Figure 6C).** Carine Joffre showed that, after 16 hours, when the cells were totally detached, there was still a significant reduction in pan β1-integrin expression at the cell surface, though only by 18% ($p<0.01$) (**Appendix Figure 6D**). Interestingly, a much more significant reduction (almost 60% $p<0.001$) was detected in cell surface levels of β1-integrin in the activated conformation (detected with anti-β1 antibody clone 9EG7) (**Appendix Figure 6E**). The reduction of activated β1 relative to pan β1 was 55 % ($p<0.01$) (**Appendix Figure 7A**) meaning that the internalised pool of β1- integrin is greatly enriched in activated β1-integrin. Immunofluorescence studies were performed with cells seeded on coverslips coated with Poly-L-lysine, which allowed rounding of tetracycline treated cells but prevented their detachment. After 5 hours of tetracycline induction, Met and β1-integrin colocalised in intracellular vesicles (**Appendix Figure 7B**).

To compare the localisation of the active β1-integrin in cells expressing or not Met-GFP, non-transfected cells and cells stably transfected with the Met-

GFP construct were seeded together and induced with tetracycline for 16 hours. In non-transfected cells, the active β1-integrin was distributed at the plasma membrane and in intracellular pools. In tetracycline-induced cells, the staining had decreased at the plasma membrane but had increased in intracellular pools, indicating again an increase in β1-integrin internalisation (**Figure 6D**). Treatment with the Met inhibitor SU11274 restored localisation of active β1-integrin at the plasma membrane (**Figure 6D**). Furthermore, triple colocalisation with EEA1 could be detected, indicating Met and β1 were recruited to EEA1 positive early endosomes (**Appendix Figure 7C**).

Together, these results indicate that, in Met-GFP expressing cells existing in suspension, Met activity promotes endocytosis of active β1-integrins and that they co-traffic through early endosomes.

The observed colocalisations between endosomal Met-GFP and active β1-integrin suggested that, as in β1A cells, β1-integrin may have an input in activated Met-GFP endosomal signalling. Consistent with this notion, siRNA mediated silencing of β1−integrin led to a significant (44%) decrease in tetracycline-induced ERK1/2 phosphorylation ($p<0.01$) (**Appendix Figure 8AB**), while Met activation and expression (**Appendix Figure 8ACD**) were not modified significantly. Thus, in both cell models, β1-integrin plays a role in the activation of Met dependent ERK1/2 phosphorylation on the endosome, under both adherent and non-adherent conditions.

3) β1−integrin is required for Met dependent anchorage independent growth, *in vivo* tumourigenesis and metastasis.

Due to the fact that the influence of β1-integrin on Met endosomal signalling occurred in non-adherent cells, with the signal being maintained for longer periods under non-adherent conditions, I investigated whether β1-integrin could play a role in Met dependent anchorage-independent growth of the normally adherent GD25, β1A and β1A-YYFF cell lines. While GD25 and β1A cells formed colonies in soft agar, HGF increased the size of β1A colonies significantly but had no effect on colonies formed by GD25 or β1A-YYFF cells (**Figure 7**). These results show that β1-integrin, and the NXXY motifs in its cytoplasmic tail, play a role in anchorage independent growth stimulated by Met.

I next wanted to test the role of β1-integrin in Met dependent tumourigenesis in nude mice using the model I have previously used; NIH3T3 cells expressing the Met oncogenic mutant M1268T (see Chapter II).

First of all however, I investigated if β1-integrin is required for the Met dependent transformation of these cells *in vitro*. Confocal analysis of Wt and M1268T Met expressing cells revealed colocalisation between Met and β1-integrin both at the plasma membrane and in EEA1-positive vesicles in M1268T Met expressing cells, while colocalisations mainly occur at the plasma membrane in Wt Met expressing cells **(Figure 8A)**. This promising result led me to perform Transwell migration assays with the Wt and M1268T

expressing cells following β1-integrin knockdown. Abrogation of β1-integrin expression **(Figure 8B)** significantly reduced the migration of M1268T cells by 79% ($p<0.01$), while there was no effect on the Wt cells **(Figure 8C)**. When these cells were grown in soft agar, there was also a significant decrease, of 44%, in the number of M1268T colonies that formed compared to cells transfected with control RNAi ($p<0.05$), whereas there was no effect on the anchorage independent growth of Wt cells **(Figure 8D)**.

As a consequence of these encouraging results, M1268T Met expressing cells were injected subcutaneously into nude mice (by Professor Ian Hart). Here I used mutant cells transfected with control- or β1-integrin-siRNA. Tumours formed rapidly in both conditions (at day 3 and 4) but tumour volumes were reduced significantly, by 50 to 60% ($p<0.001$ to 0.05), for β1-integrin silenced cells, as compared to control siRNA transfected cells, from day 5 to 11. By day 11, the tumour volumes of control silenced cells had reached $300mm^3$ and all mice were killed (**Figure 9A**). At the end of the experiment, I removed and weighed the tumours. Tumours formed by the β1-integrin silenced cells were 2.5 x smaller than those formed by their control counterparts ($p<0.05$) (**Figure 9B**).

To assess the influence of β1-integrin on Met dependent metastasis, I performed an experimental lung colonisation assay, developed with the same cells, as previously[159]. Control and β1-integrin knock down cells were injected (by Prof. Ian Hart) into the tail veins of nude mice. At 21 days, the lungs of mice injected with control siRNA transfected cells presented an average of 8.5 macroscopic tumours per mouse while this number was only

2.8 in β1-integin knocked down cells ($p<0.05$) (**Figure 9CD**). Histological analysis confirmed that while most of the lung tissue of mice injected with control siRNA transfected cells was invaded by these cells, the lung tissue of mice injected with siRNA transfected cells was largely free of metastatic involvement (**Figure 9E**). These experiments indicated that β1-integrin plays a role in-Met dependent tumourigenesis and lung tumour colonisation capacity.

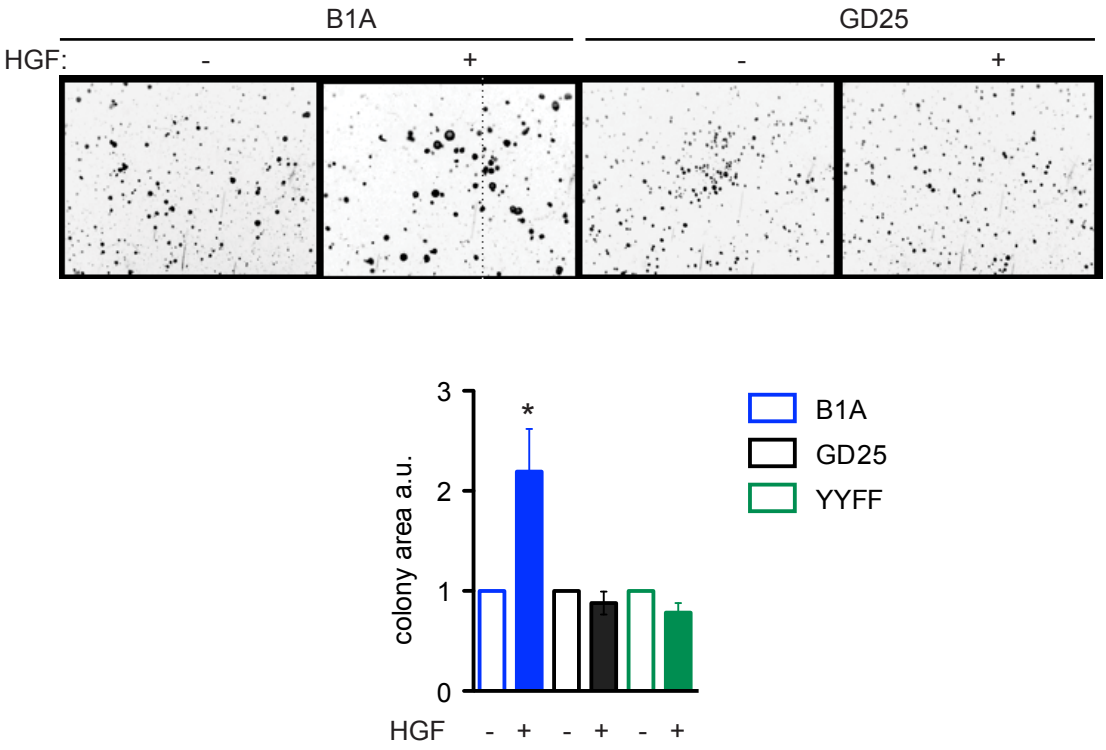


Figure 7: β1-integrin is required for Met dependent anchorage independent growth

Pictures of soft agar cultures for β1A and GD25 cells, at Day 13. From Day 8, HGF (14ng/ml) was added to the medium, or not, every other day. Data are the average colony areas ± SEM (a.u. arbitrary unit, n=3) for β1A, GD25 and β1A-YYFF cells, each experiment was done in duplicate.

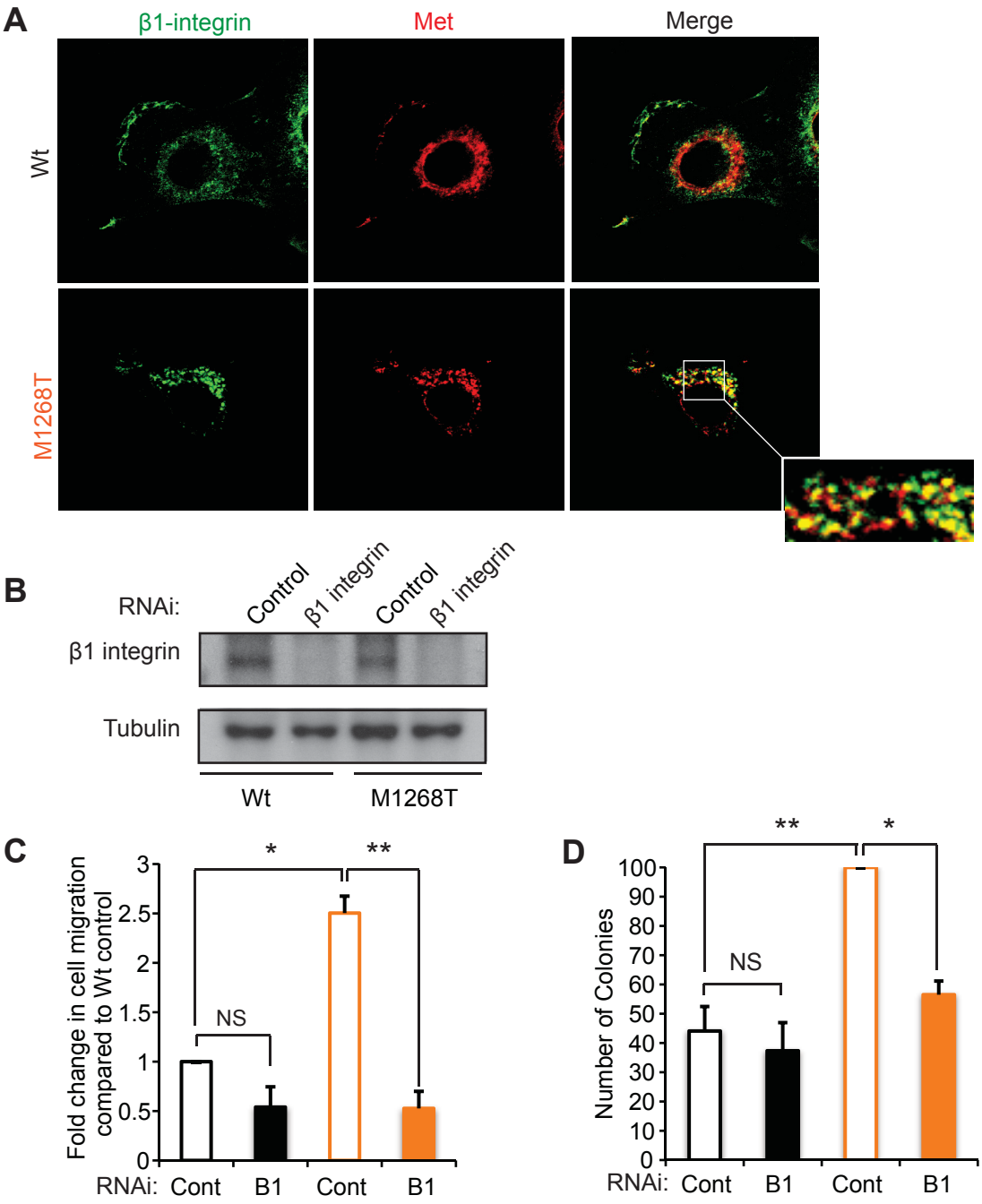


Figure 8: β1-integrin is required for M1268T Met dependent *in vitro* tumourigenicity

A) Confocal sections of Wt and M1268T expressing cells stained for Met (red), β1-integrin (green) and EEA1 (blue). Colocalisations appear in yellow. **B)** Western blots for β1 integrin and tubulin on Wt and M1268T cell lysates 72 hours following transfection with control or β1 RNAis. **C)** Fold change in Wt or M1268T Met expressing cells that have migrated through Transwells when transfected with Control or β1 RNAi (n=3), normalised to Wt control expressing cells. Each experiment was done in triplicate. **D)** Wt, and M1268T cells were grown in soft agar following transfection with control, Met or β1 RNAis. The graph represents the average colony number on Day 6. Data are mean ± SEM (n=3), each experiment was done in duplicate). *p<0.05; **p<0.01; NS=not significant.

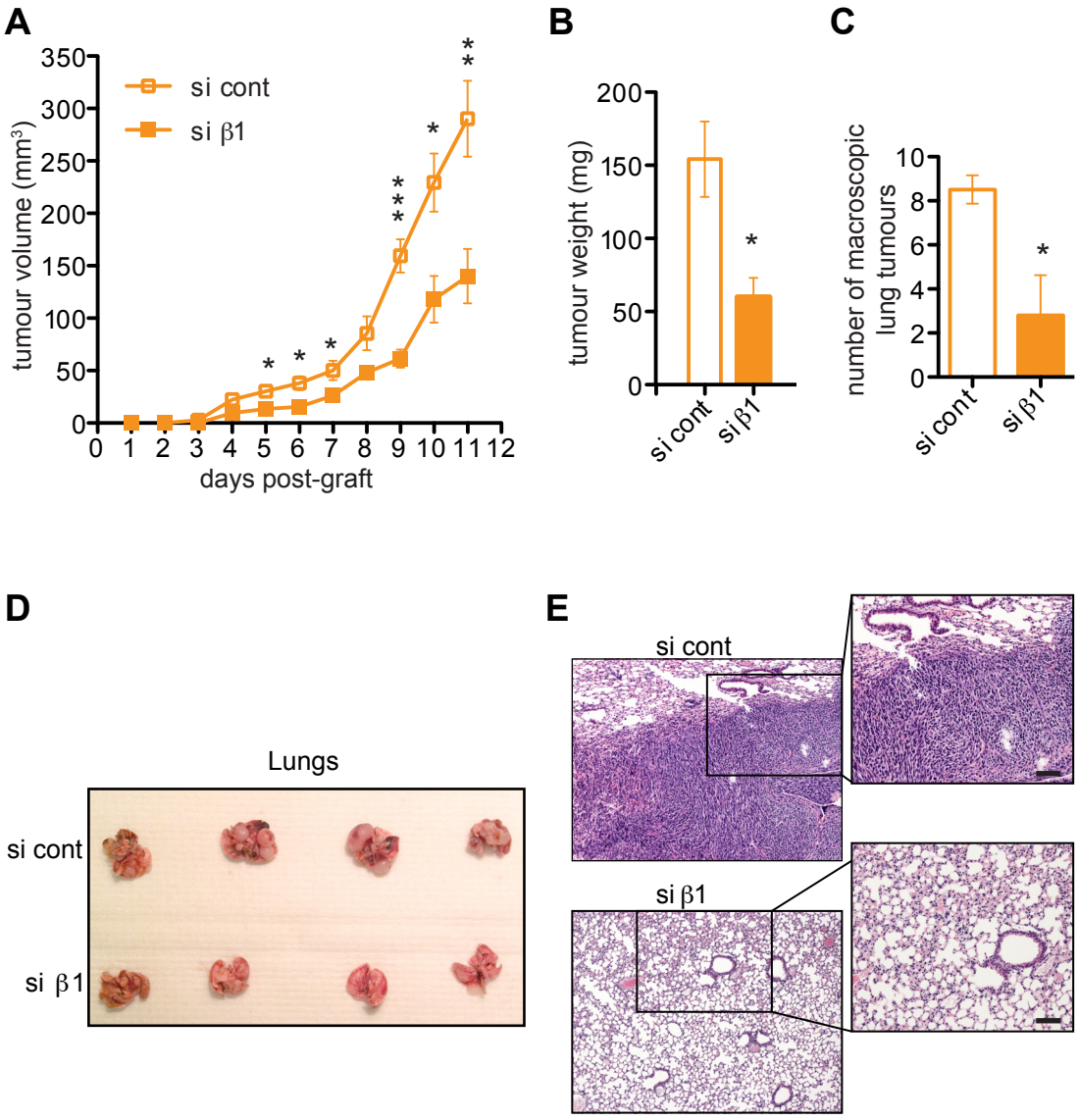
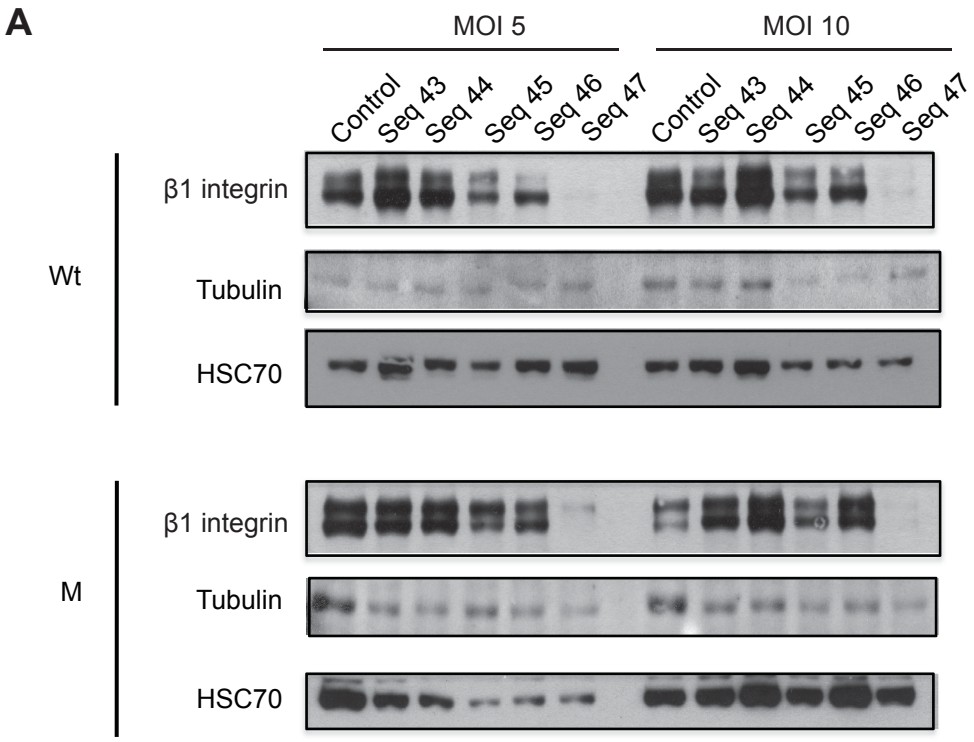


Figure 9: β1-integrin is required for Met dependent *in vivo* tumourigenesis

A) Tumour growth curves, over time, of M1268T Met expressing cells, knocked down with control or β1-integrin siRNA. Data are mean ± SEM of n=5 mice per group. **B)** Weight (mg) of tumours derived from M1268T Met expressing cells transfected with control or β1-integrin siRNA, at day 11. Data are mean ± SEM (n=5 mice per group). **C)** Number of macroscopic tumours in lungs of mice, previously injected into the tail vein with M1268T Met expressing cells, transfected with control (n=4) or β1-integrin (n=5) siRNA. Data are mean ± SEM. **D)** Pictures of the lungs of mice injected in the tail vein with either control or β1-integrin siRNA transfected M1268T cells. *p<0.05; **p<0.01; ***p<0.001.



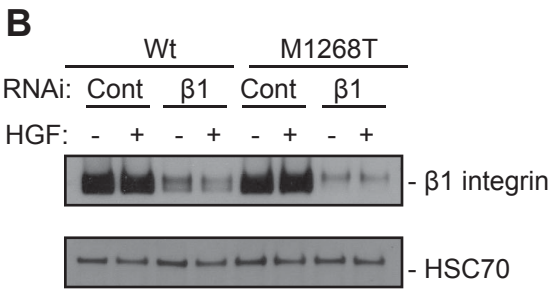


Figure 10: Development of stable cell lines lacking β1-integrin
A) Wt and M1268T cells were transduced with non-targeting control shRNA or 5 different sequences of β1-integrin shRNA (43-47) at MOI 5 and MOI 10. Knock-down was tested by western blot for β1-integrin, HSC70 and tubulin.
B) Western blot for β1-integrin and HSC70 in Wt and M1268T cells transduced with non-targeting control or sequence 47 of β1-integrin shRNA at MOI 10 -/+ HGF (50ng/ml) for 15 minutes.

Although we have previously shown (in Chapter II) that the tumorigenesis of the M1268T Met cells is dependent on Met activity, in vivo timourigenesis experiments with Wt Met expressing cells silenced for β1-integrin performed in parallel, would provide a good control. However, as Wt Met expressing tumours form much later, we can not use RNAi as β1-integrin knockdown would be too short-lived.

To overcome this problem, I have developed Wt and M1268T Met expressing cell lines that have been transduced with either Control or β1-integrin shRNA to form cell lines that permanently contain or lack β1-integrin. Following transduction of the two cell lines with 5 different shRNA sequences, it was clear that one sequence (sequence 47) was much more efficient than the others at depleting β1-integrin expression **(Figure 10A)**. I therefore expanded these cells, in which a very good knockdown was achieved **(Figure 10B)**. These results are a promising indication for future work with these cells, in which I will investigate to a greater extent the requirement for β1-integrin in Met-dependent signalling, as well as the *in vivo* tumourigenesis.

DISCUSSION CHAPTER III

β1-integrin - Met Crosstalk: an endosomal inside-in signalling

This study demonstrates that Met activity (either constitutive or HGF induced) promotes Met and β1-integrin internalisation and co-recruitment on endosomes. From this location, β1-integrin plays a role in Met downstream signalling, leading to an optimal and sustained ERK1/2 activation. The intact NXXY signalling motifs in the β1-integrin cytoplasmic tail appear to be required since the Y to F mutations lead to a comparable reduction in HGF-induced ERK1/2 phosphorylation to that observed in the absence of β1-integrin. Interestingly, this influence of β1-integrin on Met signalling occurs in cells existing both under adherent and non-adherent conditions. Consistent with these results, we also show that, in addition to playing a role in Met dependent cell migration, β1-integrin signalling is required for Met stimulated anchorage-independent growth, *in vivo* tumorigenesis and experimental metastasis.

1) Met activation triggers Met and β1-integrin co-internalisation

β1-integrin trafficking is altered by Met activation

β1 trafficking (cycles of endocytosis and recycling) recently has been shown to play a large role in cell migration[231, 248, 316, 317]. Interestingly, using three different cell models, we have found that upon Met activation, by both HGF dependent and independent mechanisms, β1-integrin is internalised and coincidently the presence of β1-integrin is required for Met dependent cell migration.

The fact that the GFP-Met cells detach upon tetracycline induced expression of the constitutively active GFP-Met may be due to internalisation of β1-integrin induced by Met activation.

β1-integrin was recently reported to internalise both in inactive and active conformations. Moreover, the net endocytosis rate of the active β1-integrin is higher, whereas the inactive β1-integrin recycles back to the plasma membrane[241]. Consistent with this, our data show that Met activity increases β1-integrin internalisation and that an important part of this internalised integrin is in the active conformation.

Thus our hypothesis is that, to play a role in Met signalling on the endosome, β1 needs to be in an active conformation.

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Met and β1-integrin co-internalise

We have observed Met - β1-integrin colocalisation on endosomes upon Met activation. This, together with the observation that the two molecules already colocalise at the plasma membrane in cells with inactive Met, suggested to us that the two molecules co-internalise. Ludovic Menard in the lab has now confirmed this by real time confocal microscopy (not shown). We are currently performing co-immunoprecipitation and PLA studies to assess direct/indirect association between the two molecules.

2) β1-integrin is required for Met endosomal dependent signalling in anchored and unanchored cells

β1-integrin is required for Met dependent signalling even in cells in suspension

Here, we have shown that β1-integrin plays a role in Met signalling. Interestingly, β1-integrin expression recently was reported to also be required for full EGFR downstream signalling upon EGF stimulation in the lung cancer cells A549 [320]. It is worth noting however that β1-integrin silencing increased the expression of EGFR on the cell surface, something we have not observed for Met but we will look at this more carefully in future experiments. Interestingly, we are currently extending our study to human cancer cell lines including A549 cells, which will allow us to compare the mechanisms involved for these two RTKs (EGFR and Met), in the same experimental conditions.

It may appear not so surprising to find that an RTK, such as Met, and an integrin, such as β1-integrin, cross-talk. This concept has been described for many RTKs, including Met[258, 260, 261]. However, most cases of these cross-talks were reported to occur upon cell attachment, thus upon integrin ligand binding. What is most surprising about our study is that the cross-talk occurs in both attached cells and cells in suspension. This suggests that, here, β1-integrin has an unusual role, independent of its ligand binding functions. In fact, such a role for β4-integrin in Met signalling has been described by Trusolino et al.[258]. They showed, in attached cells, that β4-integrin acted as a signalling scaffold molecule to enhance Met signalling rather than as an adhesion receptor.

Thus our study is the first to demonstrate a requirement for β1-integrin in Met signalling, that moreover occurs in detached cells (**Figure 11**).

Met signalling require a fully functional NXXY motif in the cytoplasmic domain of β1-integrin

We have shown that optimum and sustained ERK1/2 signalling upon Met activation require the presence of the two tyrosine residues in the NXXY motif in the β1-integrin cytoplasmic tail, Y783 and Y795. This motif has been previously been implicated in integrin function, including regulation of integrin activation[231] and to be critical for β1-integrin mediated activation of FAK[319].

Thus our results suggest that β1-integrin acts as a scaffold for Met signalling to the ERK1/2 pathway in a similar way to β4-integrin, which was reported to function as a scaffold between Met and ERK1/2 and Src, through binding Shc and Shp2 respectively[258, 259]. Thus, future experiments for this project will consist of investigating co-localisations and co-immunoprecipitations between Shc and Met as well as Shc phosphorylation upon Met activation in the presence and absence of β1-integrin with its functional or mutated NXXY motif.

3) The cross talk appears to occur on endosomes: an "Inside-in signalling"

One important hypothesis in our study is that we believe the cross-talk occurs on endosomes. The observation that, even in cells in suspension, Met and β1-integrin co-localise on endosomes and that β1-integrin is required for maximum and sustained ERK1/2 activation strongly suggests that β1-integrin impinges on Met signalling directly on endosomes, hence the term "inside-in

signalling". Especially due to the fact that ERK1/2 activation upon Met activation has previously been shown to require Met endocytosis[62]. In the above reported Met-β4-integrin[258] and EGFR-β1-integrin[320] cross-talks, the location of the RTK and the integrin was not investigated.

I will perform further studies to check our hypothesis. In particular, I will ask whether blocking β1-integrin internalisation, while maintaining Met endocytosis, leads to a significant reduction in Met dependent ERK1/2 phosphorylation. We plan to achieve this using Rab21 knockdown by RNAi[321-323]. I already have checked that Rab21 knockdown does not affect Met internalisation (data not shown).

Furthermore, blocking the internalisation of each molecule separately will determine whether it is the presence of both, or of only one, that is required on endosomes. As we saw in chapter II, Met internalisation could be blocked using c-Cbl or Grb2 inhibition or alternatively using Met mutated in the Grb2 binding site [159].

One could argue that rather than β1-integrin being important for Met endosomal signalling, the two molecules just need to be at the same place in order to interact. Thus we could imagine that when both are maintained at the plasma membrane, Met signalling would not be affected. This is however unlikely as we already know that blocking dynamin using for example Dynasore, which we have shown to block Met endocytosis in several cell systems (see Chapter I)[157, 159] and should also block β1-integrin, does inhibit

Met dependent ERK1/2 signalling. However, we will verify this in the cells studied here.

Due to the fact that it is predominantly the active form of β1-integrin that appears to preferentially traffic upon Met activation and colocalise with Met on endosomes, future experiments will involve investigating cells lacking Sharpin, an endogenous inhibitor of β1-integrin activity[324]. These cells have a 2-4 fold increase in the active form of β1-integrin, even when cells are in suspension[324]. Thus it will be very interesting to investigate Met-dependent β1-integrin localisation as well as the effects on Met signalling in these cells compared to cells expressing normal levels of Sharpin.

The term “inside-in signalling” has been coined here, due to the fact that this novel mode of signalling appears to occur on endosomes and is cell anchorage independent, in marked contrast to the “outside-in” and “inside-out” signalling mechanisms[228, 231, 233]. “Outside-in signalling” occurs when integrin ligand binding, increases interactions with the cytoskeleton[231, 232]. While “inside-out signalling” occurs when changes in the cytoskeleton alter the ligand-binding affinity of integrins[228, 231].

Interestingly, the reduction in EGFR signalling upon β1-integrin knockdown observed in the recent study by Morello et al., could be partially rescued by over expression of Rab Coupling Protein (RCP)[320], suggesting that integrin recycling may be important. It may be interesting to investigate the role of

RCP in Met- β1-integrin cross-talk in our models, especially given the recent evidence that RCP is involved in recycling of both Met and α5β1-integrin[262].

4) Biological outcome of Met-β1-integrin cross-talk

Role for Met-β1-integrin cross-talk in cell anchorage independent growth

Despite detachment from the plate upon tetracycline induction, GFP-Met cells do not undergo anoikis. In fact the expression of Met gives them a survival advantage versus HEK293 cells lacking Met expression in suspension as assessed by flow cytometry (not shown). We plan to determine whether β1-integrin silencing reduces the survival of these cells.

Moreover we find that β1-integrin expression increases the ability of both the GD25 and M1268T Met expressing cells to undergo anchorage independent growth upon Met activity. Similarly, the group of Comoglio has shown that, consistent with its role in Met signalling, β4-integrin was necessary to sustain Met dependent anchorage independent growth[258, 259].

Met dependent cell migration and anchorage independent growth require a fully functional NXXY motif in the cytoplasmic domain of β1-integrin

Met dependent cell migration as well as anchorage independent growth both were found to require the presence of the two tyrosine residues in the NXXY motif in β1-integrin cytoplasmic tail, Y783 and Y795. To investigate further the role of this β1-integrin motif in Met signalling and Met dependent biological outcome, I plan to stably express the human Wt or M1268T Met

constructs in Wt and β1-integrin-YYFF murine embryonic fibroblasts (MEFs)[325], obtained from our collaborator in this study, Prof. Johanna Ivaska. In these cells, I will perform studies *in vitro* and *in vivo* as detailed in Results Chapter III-3.

A role for the endosomal Met-β1-integrin cross-talk in cancer?

The role of Met in tumour formation and progression has been demonstrated in many types of cancer, including bladder, breast, liver, lung, prostate, pancreas and colorectal cancer[8]. Meanwhile the lack of β1-integrin expression in a mouse model of breast cancer (MMTV/PyV MT) decreased tumour growth[326]. The same was observed in a mouse model of pancreatic cancer (Rip1Tag2), where additionally metastasis also was reduced[327]. Thus both of these transmembrane receptors have established roles in cancer formation and metastasis.

As both Met and β1-integrin play important roles in tumourigenesis and tumour progression, it seemed logical that the two molecules would cooperate to enhance each other's signalling. Thus, I have shown that lack of β1-integrin in the established M1268T Met driven *in vivo* tumourigenesis model [159] (see Chapter II) significantly reduces tumour growth and experimental metastasis. Importantly however, as I explained in the results part, I am planning to repeat these experiments using β1-integrin shRNA (instead of siRNAs). This will allow me to compare the influence of β1-integrin silencing between the mutant and the WT cells and thus, confirm that β1-integrin plays a role in Met dependent tumorigenesis.

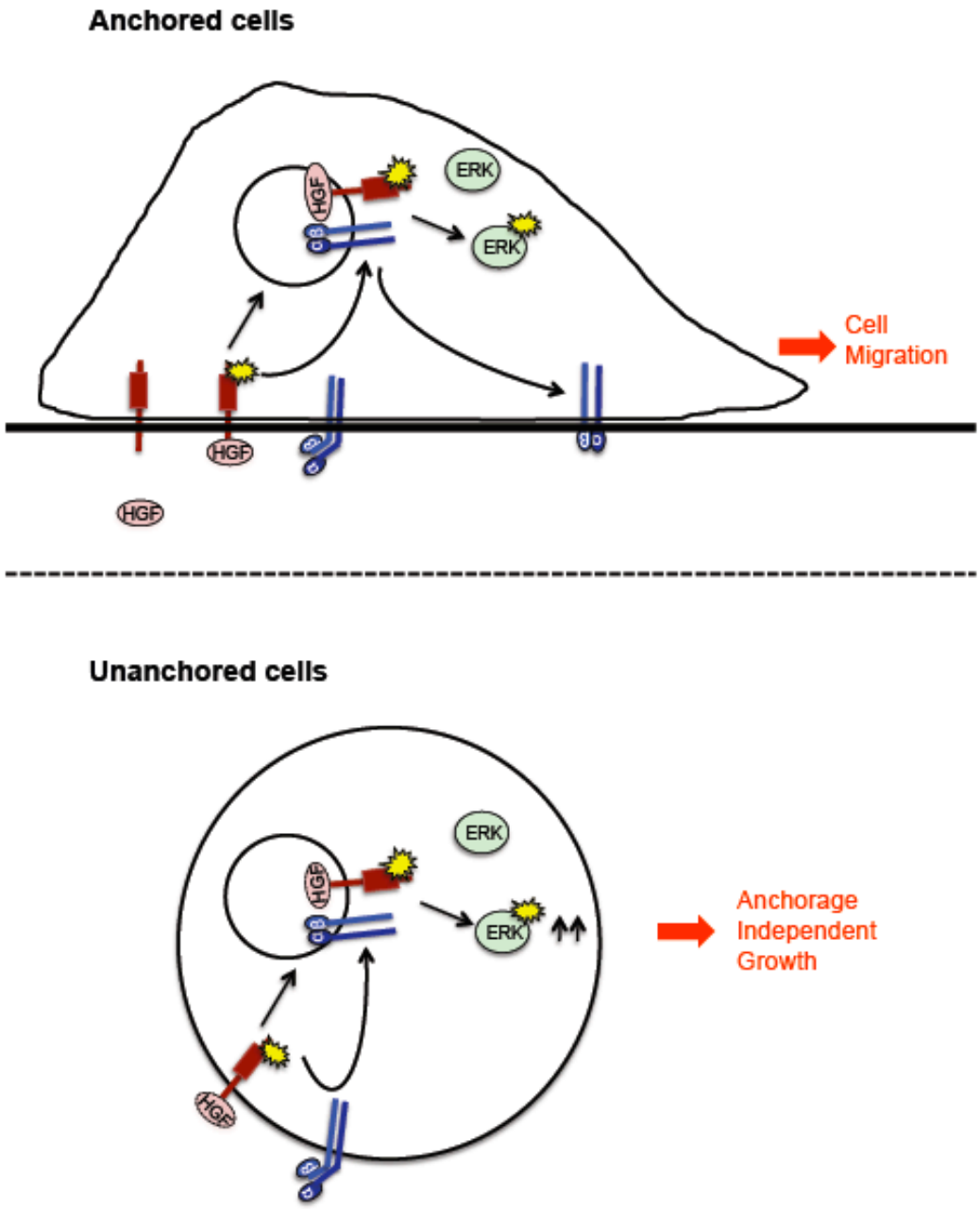


Figure 11: Model of Met- β1-integrin cross-talk in anchorage dependent and independent conditions.

In anchored cells and unanchored cells, activation of cell surface Met leads to the internalisation of Met and β1-integrin. They are co-recruited on endosomes, and they cooperate to promote HGF dependent ERK1/2 activation. In anchored cells, activated ERK1/2 plus the recycling of β1-integrin to the cell leading edge triggers cell migration. In unanchored cells, sustained activated ERK1/2 stimulates anchorage independent growth.

We currently are extending our study in human cancer cell lines including breast and lung. So far, our preliminary data suggest that the endosomal Met-β1-integrin cross-talk also occurs in these cells. For example, in the MDA-MB-468 cells, we have observed a co-internalisation of Met and β1-integrin and silencing β1-integrin reduces Met dependent ERK1/2 phosphorylation.

Interference with RTK-Integrin cross-talk as a treatment option?

β1-integrin is an ubiquitously expressed integrin and broad inhibition of β1-integrin may therefore have severe consequences on cells. Although it is worth noting that, in our experiments, knockdown of β1-integrin, both transiently and stably, does not appear to affect cell growth or proliferation in culture. In fact, there are a few β1-integrin inhibitors available, that have already undergone clinical trial testing. ATN-161 and JSM6427 are both α5β1-integrin antagonists, which inhibit the interaction between α5β1-integrin and fibronectin. JSM6427 was first described in 2006, when it was demonstrated to decrease ERK1/2 phosphorylation that can occur upon fibronectin binding as well as induce apoptosis in endothelial cells[328]. Furthermore, it has been shown to reduce glioma growth *in vivo*[329]. JSM6427 has recently completed a phase I clinical trial, although the results are as yet unknown (www.clinicaltrials.gov). ATN-161 is a small peptide consisting of 5 amino acids and in animal models it has been shown to prevent tumour growth and metastasis both alone and in combination with chemotherapy or radiotherapy[330]. Importantly, a phase I clinical trial revealed low toxicity and prolonged stable disease in 1/3 of participants with advanced solid tumours[330]. Consequently, a phase II clinical trial is being set up in head and

neck cancer patients combining ATN-161 and chemotherapy or radiotherapy[330]. Volociximab, also known as M200, is a humanised monoclonal antibody against α5β1-integrin[330]. Phase I clinical trials have been successful with low levels of toxicity and stable disease occurring in a quarter of patients with advanced solid tumours[330]. There are currently 13 phase I/II clinical trials of Volociximab that are recruiting, ongoing or have recently been completed in a variety of conditions including cancer (www.clinicaltrials.gov). The various inhibitors of β1-integrin therefore appear to be quite successful, perhaps because they target a single heterodimer, α5β1-integrin. They may indeed be valuable as a potential anti-cancer treatment and also it has been suggested that they may inhibit breast cancer recurrence[330, 331].

Taking our results into consideration, an interesting question would be whether such β1-integrin inhibitors could be used to treat Met dependent cancers? Our *in vivo* experiments suggest this **(Figure 9)**. Moreover, our future planned experiments using Wt or M1268T Met expressing cells stably lacking β1-integrin expression may confirm this further and reveal whether there is any compensation by other integrins as well as the effects on Met dependent tumourigenesis *in vivo*.

Alternatively, more knowledge of the way in which specific RTKs and integrins interact and traffic may provide us with a way to specifically inhibit the interaction or perhaps the localisation of specific RTK-integrin cross-talk that is driving tumourigenesis.

CONCLUSION

"Endosomal signalling" of Met and cancer

Traditionally, the concept was that receptors signal from the plasma membrane only and then get internalised to be "switched off" through degradation. **However my results show that the RTK Met can still signal post-endocytosis in several different cell models. Moreover I have demonstrated that an endosomal location of Met plays a role in tumourigenesis**.

Firstly, I found that Met is expressed, activated and traffics in a panel of human breast cancer cell lines. Importantly, aggressive breast cancer cell lines appear to have altered Met trafficking and degradation, suggesting a longer time spent on endosomes. Furthermore, the aggressive MDA-MB-468 cell line has a larger dependence on Met endocytosis for the Met dependent signalling and cell migration than a model of DCIS. Supporting these experimental observations I was able to show, in a pilot study on human breast cancer tissues, that Met was found to exist increasingly in an endosomal location as breast cancer progresses. This combination of cell line and clinicopathological data suggests that Met endosomal signalling might play an important role in breast cancer progression.

Secondly, using a model of Met oncogenic mutants, I showed that Met endocytosis occurs via dynamin, clathrin, c-Cbl and Grb2 –dependent

mechanisms. In addition, I found that HSP90 protects the Met mutants from degradation. Importantly, either inhibition of endocytosis or restoration of degradation through targeting the newly discovered mechanisms, reduced the accumulation of mutant Met on endosomes. As a result, the transforming capacities of the mutants were greatly reduced *in vitro* and *in vivo*, including that of a Met mutant found to be resistant to Met inhibitors. Thus, the endocytosis of these Met mutants was found to be directly linked to their tumourigenicity. Such that it is its accumulation on endosomes coupled with constitutive activation that leads to tumour formation and metastasis.

Finally, I have discovered that β1-integrin may act as a scaffold for Met endosomal signalling in several cell models. Met activity promotes Met and β1-integrin internalisation and co-recruitment on endosomes. Here, β1-integrin, through its NXXY motif, is required for optimal ERK 1/2 activation in anchored but, more surprisingly, also in unanchored cells. Furthermore, I have also shown that, in addition to playing a role in Met dependent cell migration, β1-integrin signalling is required for Met stimulated anchorage-independent growth and *in vivo* tumorigenesis. We propose that β1-integrin promotes Met signalling through a novel mechanism that we called "endosomal inside in signalling".

It is currently becoming clear from various recent studies that the trafficking of RTKs and their "endosomal signalling" play a major role in cellular outcomes. This, together with the emerging knowledge that many trafficking proteins, including Hip1 and Rab25[332, 333], have altered expression in cancer,

strongly implies that trafficking and more specifically “endosomal signalling” could play an important role in cancer formation and metastasis.

Various signalling pathways that are involved in cancer have been shown to be generated on endosomes. For instance, the endosomal signalling of EGFR is sufficient to activate ERK1/2[144]; Rac1 also is activated on endosomes upon HGF stimulation[158] and both STAT3 and ERK1/2 require Met endocytosis for their full activation[62, 157]. The endocytosis of RTKs has also been shown to be important in cell functions including cell migration[149, 150, 158] and cell survival[144, 162]. However, until our study on Met mutants was published in 2011[159] nothing had previously shown that the altered endocytic trafficking of RTKs can directly be involved with the development of cancer.

A possible reason for the role of Met endocytic signalling in cell transformation is that the strength of Met and its downstream signalling may be maintained to a greater extent on endosomes. Endosomes may provide a better proximity of Met molecules with each other, which could be important for maintaining a better trans-autophosphorylation and/or a reduced access to phosphatases. Additionally, Met “endosomal signalling” may provide control over the specificity of Met dependent signals due to the fact that endosomes are able to act as signalling platforms bringing together various components for either the initiation or prolongation of signalling both spatially and temporally[160, 162, 334]. It is therefore possible to see how cancer cells may alter their trafficking in order to exploit specific signalling pathways.

The publication of studies demonstrating that mutant p53 not only leads to a loss of tumour suppressor function, but also has a gain of function effect by altering RTK and integrin trafficking[255, 262], is of great interest to the cancer research community, as huge number of cancer patients (50%) have p53 mutations[255, 262 254]. These studies showed that mutant p53 increases recycling of α5β1-integrin and EGFR[255] or Met, in basal conditions, and increases signalling of these RTKs[262]. However, these studies did not investigate a potential co-trafficking of the RTK and α5β1-integrin upon HGF stimulation. In their model, whether the resulting tumourigenesis and metastasis is due to an increased life-span of the receptors, the recycling of individual receptors or the co-trafficking and interaction between these two classes of receptors is unknown.

However, results of these studies and our study are not necessary exclusive, but more work is required to find out whether the two mechanisms co-exist in the same cancer cells. In our study we suggest that an accumulation of Met and β1-integrin on endosomes aids Met signalling and this may therefore also be the case for the studies performed on cells with mutant p53. Interestingly, the aggressive breast cancer cells studied in Chapter I; MDA-MB-468 and MDA-MB-231, both have mutant p53[283] and it is in these cells that I have observed a small amount of Met recycling. Thus the cross-talk on endosomes between β1-integrin and Met in these cells is something which would be very interesting to investigate.

Potential prognostic and therapeutic implications of my findings

Met in cancer

Several studies have reported an increased Met expression in various human cancers including breast cancer[174, 175] and that Met is a marker of poor prognosis. Potentially, one of the reasons for this is that Met can activate many downstream signalling pathways leading to the initiation of many cellular functions. In fact Met can initiate many of the "Hallmarks of cancer" originally described by Hanahan and Weinberg in 2000[57], including cell survival, growth, invasion and angiogenesis[8].

In fact, the M1268T mutant, investigated in this thesis, has been shown to trigger the development of basal-like breast tumour in mice [182]. This, together with the recent literature suggesting Met as a marker for basal breast cancer[175, 277], suggest a role for Met activation in the development of basal breast cancer.

Several studies indicate that Met could represent not only a prognostic factor but also possibly a target for therapy. As a consequence, Met is now established as an important target to consider for anti-cancer therapies, a fact that is demonstrated by the many Met inhibitors that are currently undergoing clinical trials (see Chapter II-1).

Considering the results obtained in my study, it also seems that Met "endosomal signalling" is important in the development and progression of cancer. Here we have provided a direct link between the endocytosis of a constitutively active Met mutant and tumourigenesis as well as a potential

role for endosomal β1-integrin in Met dependent tumourigenesis. We also present data that suggest that Met endosomal signalling correlates with breast cancer progression. Furthermore, this study suggests that the subcellular localisation of Met may also provide a prognostic marker, which may be important for pathologists to consider for cancer diagnosis, providing we confirm our results on a larger cohort of breast cancer tissues. Thus, the current models of oncogenic RTK driven tumorigenesis may require a rethink to also investigate the location of the RTK and the possibility of endosomal signalling.

Inhibiting Met-dependent tumourigenesis

The Met mutations studied in Chapter II are naturally occurring oncogenic mutations found in human cancers. My results suggest that patients with specific Met mutations may be resistant to small molecule Met inhibitors, highlighting the importance of knowing a patient's genetic make-up prior to treatment. As we move closer to personalised cancer treatment, with more available and cost effective genetic screening, this might be feasible in the future. It further demonstrates the need for comprehensive *in vitro* and *in vivo* studies on mutated proteins as well as the appropriate classification of patients in clinical trials. It is worth noting however, that the Met mutations I have studied are quite rare and have not been found to occur in 'major' cancers, such as breast cancer. Thus, while our work on these Met mutants is relevant to patients bearing these mutations, they predominantly provide a good model to study Met dependent tumourigenesis.

Importantly, however, my results suggest potential alternative ways to target Met in cancer that appear to both effectively reduce Met dependent tumourigenesis and overcome resistance to TKIs, in my experimental model.

My studies indicate that inhibition of endocytosis might be a potential therapeutic option for preventing the development and progression of Met driven cancers. While Met is an important target for various cancers, it is also involved in other normal biological functions such as homeostasis or wound healing[51]. Therefore, inhibiting Met endocytosis may impair the transforming signals only, according to our hypothesis that Met triggers its transforming signal from the endosome location. Thus, targeting the localisation of Met, rather than its activation, may have limited effects on the biological functions of the normal, non-transformed cells while at the same time reducing the Met-driven cell transformation.

In addition to the inhibition of Met activation or Met endocytosis, the observation that HSP90 inhibitors can reduce Met dependent tumourigenesis by effectively inducing Met degradation is very interesting. HSP90 is ubiquitously expressed and has important functions in non-tumourigenic processes[335]. However, its expression is increased in many tumours and this therefore makes it a valuable target[335]. HSP90 inhibitors have been shown to provide a promising therapeutic approach to treat many cancers, including breast cancer[336], and they are being tested in various clinical trials. They are known to target multiple oncogenic proteins that are protected by HSP90,

including various RTKs, downstream signalling molecules such as AKT and even mutant p53. In this study we have also demonstrated that HSP90 inhibitors may be used to treat Met driven cancers.

The observation that the ubiquitously expressed β1-integrin plays a role in Met endosomal signalling, suggests that β1-integrin antagonists or antibodies may be able to also target Met driven cancers. Furthermore, my studies have highlighted the need for further investigations on the mechanisms regulating RTK-integrin interactions and/or trafficking as their impairment could constitute the basis for the development of novel inhibitors against cancer development.

Would RTK localisation be a plausible target for future therapy?

A greater amount of research is required to fully understand the roles of RTK trafficking and endosomal signalling in tumourigenesis and cancer metastasis. Novel strategies are required to inhibit Met dependent tumourigenesis based on altering Met localisation or by devising "endosome-specific targeting" approaches[337], as broadly inhibiting endocytosis will have many unspecific targets and probably have high levels of toxicity, despite the fact that in our model there did not seem to be a large amount of adverse effects on our control non transformed Wt Met expressing cells. Therefore, more in-depth knowledge is required of individual RTK internalisation mechanisms for drug-development to progress.

However, this ongoing research, together with a larger investment from pharmaceutical companies in the future, may make it possible to specifically inhibit the endocytosis of individual RTKs or interactions between RTKs and integrins that occur in certain cellular locations.

It is hoped the suggested approach will represent a plausible alternative as a therapy for patients, particularly with advanced disease, in the future. It may target the dangerous signals while allowing normal cells to activate the signalling pathways that they require for normal cell functions and may also potentially target the 'harmful signals' of potentially multiple RTKs simultaneously. Interestingly, the use of agents to block Met endocytosis as well as HSP90 inhibitors to restore Met degradation, led to less accumulation of Met on endosomes and as a consequence reduced the tumourigenic properties of a Met mutant that I have shown to be resistant to conventional Met small molecule inhibitors. It is possible that control of signal location could provide an alternative therapy for those patients who are resistant to traditional RTK inhibitors.

APPENDIX

MATERIALS AND METHODS

1) Reagents

a) Antibodies

Antibodies for flow cytometry:

Rat monoclonal anti-mouse anti- β1-integrin, clone MB1.2 (MAB1997) from Millipore; rat monoclonal anti- β1-integrin in active conformation, clone 9EG7 from BD Biosciences; mouse monoclonal anti-human anti- β1-integrin, clone DF7 from Biomol International. PE-conjugated donkey anti-rat (1/250) and APC-coupled donkey anti-mouse (1/250) from Jackson Immunoresearch were used as secondary antibodies.

b) Plasmids

Met GFP construct

The human hepatocyte growth factor receptor (Met) open reading frame (ORF) was first introduced into pEGFP-N1 (BD Clontech) by Dr. Stephanie Kermorgant. Met ORF was amplified by PCR, using the flanking primers 5' - ccgctcgagatgaaggcccccgctgtgc-3'(Xho I site) and 5' - cccccaagcttcaatgatgtctcccagaaggaggc- 3' (Hind III site) (the underlined sequence represents the mutated stop codon). Plasmid containing Met-EGFP construct was then digested with Eco RI and Not I restriction enzymes and was introduced into pcDNA™ 4/TO (Invitrogen Life Technologies) containing full length wild type Met digested with both restriction enzymes by Dr. Stephanie Kermorgant. Construct was then checked by full sequencing.

2) Flow Cytometry

Expression analysis

To determine the level of the desired protein at the plasma membrane, flow cytometry was performed by Dr. Carine Joffre. Cells were split and washed 2 times in cold FACS buffer (PBS 2% serum). $1x10^6$ cells were incubated with primary antibodies in 100μl cold FACS buffer on ice for 1 hour. After incubation, cells were washed three times in 1ml of cold FACS buffer (centrifuged at 400 g for 5 minutes between each wash) and incubated on ice with PE or APC conjugates for 30 minutes. After which, cells were washed three times and then resuspended in 300μl of cold FACS buffer. Flow cytometry data were acquired on a FACS Calibur (Becton Dickinson).

3) Immunofluorescence

Colocalisation analysis.

Colocalisation analyses were performed by Dr. Ludovic Menard (cells on coverslips) and Dr. James Hulit (breast cancer tissue). Imaging parameters were set as follows; scan mode was set to frame; averaging set to 4 scans. Image dimensions at a 1.0 magnification were 134.82 microns along each of the x and y axes with images captured at a 1024x1024 resolution. Pinhole was set to 1 airy disk relative to the red emission, yielding a z axis slice of 0.9 microns.

The intensity threshold of the green and red channels were independently set to only include vesicle-like structures which were identified as areas of punctate staining. Only pixels identified as originating from a vesicle were included in the analysis, those not determined to be of vesicle origin were

excluded as noise. Imaging parameters allow us to confidently identify structures at a lower limit of roughly 200nm diameters. Data are displayed as the percent of green pixels (EEA1) with overlapping red pixels (Met).

Co-localisation analysis of Met:EEA1 was performed on human breast tissue from 4 normal, 10-15 DCIS and IDC cases, from which a minimum of 2 fields were analysed per case. The total number of cells analysed from these normal, DCIS and IDC samples were 800, 612 and 427 cells respectively.

4) Immunohistochemistry

Immunohistochemistry

Immunohistochemistry was performed by Dr. Colan Ho-Yen on DCIS and IDC TMAs. TMA sections were dewaxed in xylene and then re-hydrated through alcohol and placed in 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was performed by microwave treating the sections in 0.1M citrate buffer at pH6 for 10 minutes. After washing in PBS, normal horse serum was deposited on the sections for 15 minutes at a dilution of 1:75 in 1%BSA/PBS to block non-specific antibody binding. After draining the sections, met (mouse monoclonal antibody 8F11, Novocastra) was incubated for 1 hour at room temperature at a dilution of 1:150. Following PBS washes, the secondary biotinylated horse anti-mouse antibody (Vectastain, Vector Laboratories) was applied to the sections at a dilution of 1:200 for 40 minutes. The sections were washed in PBS then incubated with avidin-biotinylated peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 30 minutes. The reaction was developed using a DAB kit (Vectastain, Vector Laboratories), after which sections were

counterstained with haematoxylin, rinsed and dehydrated through alcohol to xylene and mounted with coverslips. A section of pancreas served as a positive control and for the negative control, the primary antibody was omitted.

Scoring

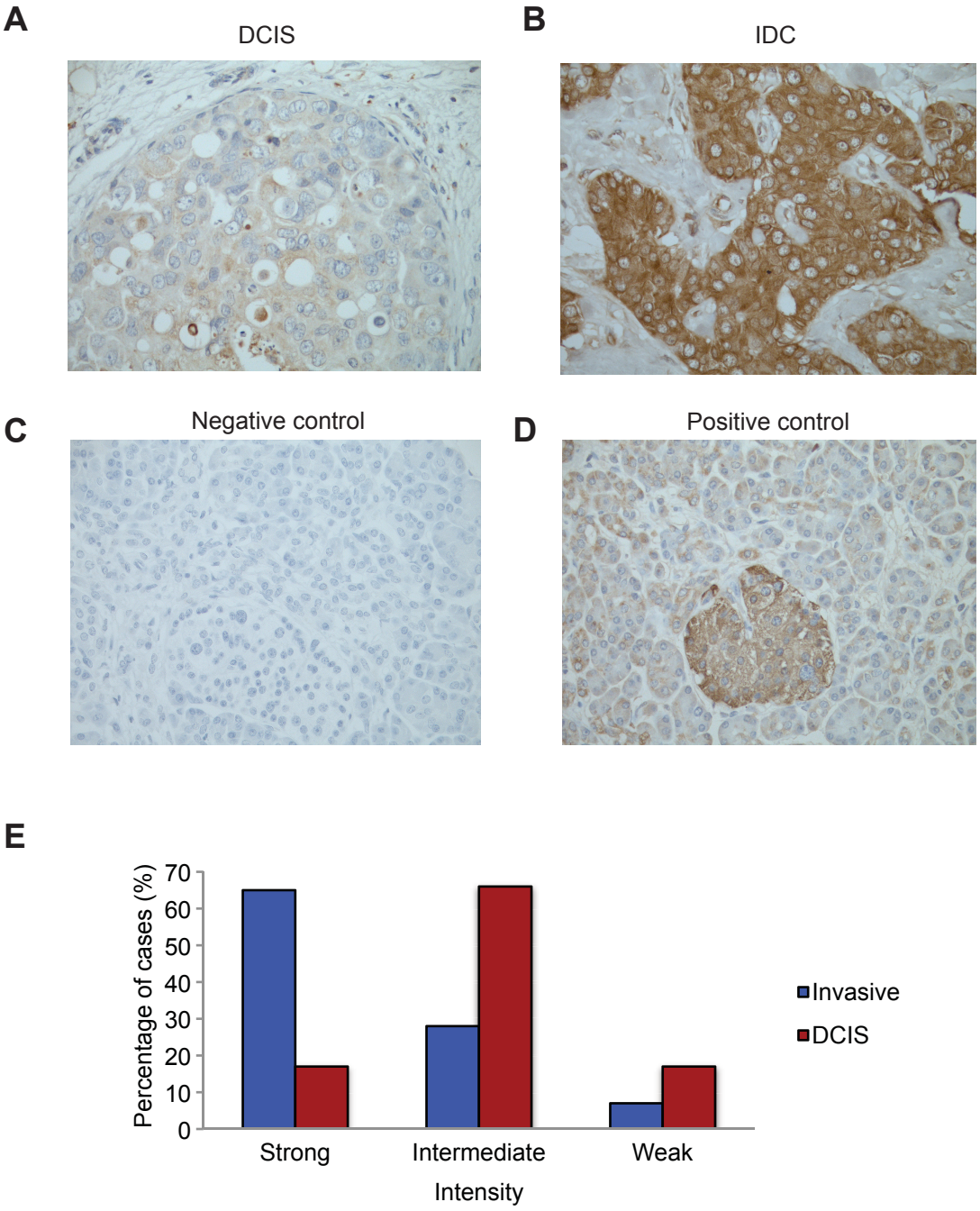
For the semi-quantitative analysis, the cytoplasmic and membranous compartments of the tumour cells were scored individually. Intensity was scored on a scale of 0-3 (0 = no staining, 3 = strong) and area of reactivity was scored on a scale of 0-4 (0 = no staining, 1 = less than 25%, 2 = 25-50%, 3 = 51-75% and 4 = more than 75%). The intensity and area were combined to give a score out of 7 and cytoplasmic and membranous scores were then added to give a total score out of 14. The total score was categorised as follows: 0-4 = weak, more than 4 but less than 10 = intermediate and greater than 10 = strong.

5) Cell Transfection

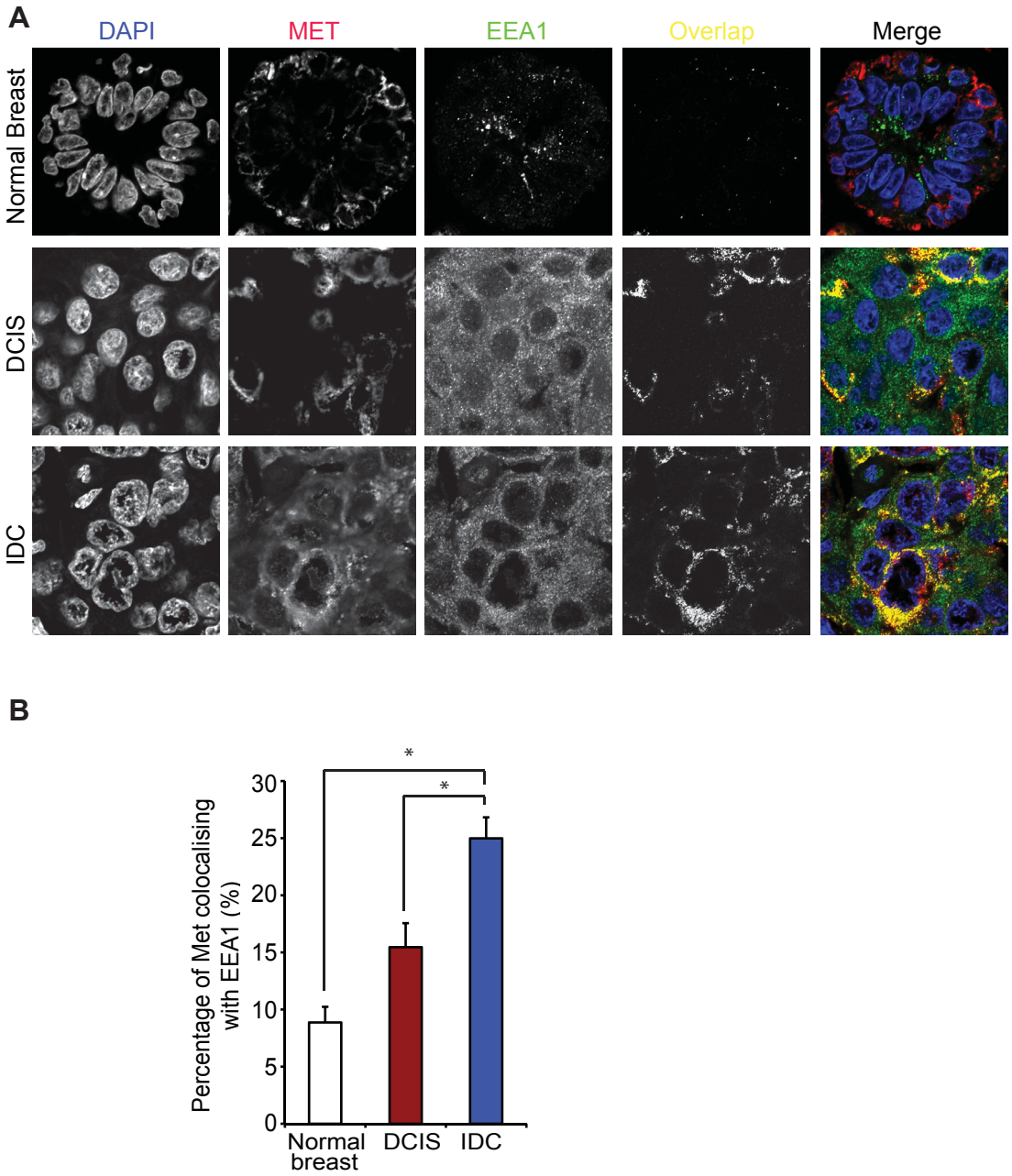
Oligofectamine transfection

Met-GFP cells were transfected using Oligofectamine. Cells were plated at $1x10^5$/well in six-well plates. 24 hours later cells were transfected in 1ml serum-free Optimem (Invitrogen life technologies). 180ul optimem + 10 μl of 20mM RNAi was mixed with 16ul + 4ul oligofectamine (Invitrogen Life Technologies) having been prepared separately and incubated for 7 minutes. 200ul of the transfection mixture was added to each well. 1ml of complete

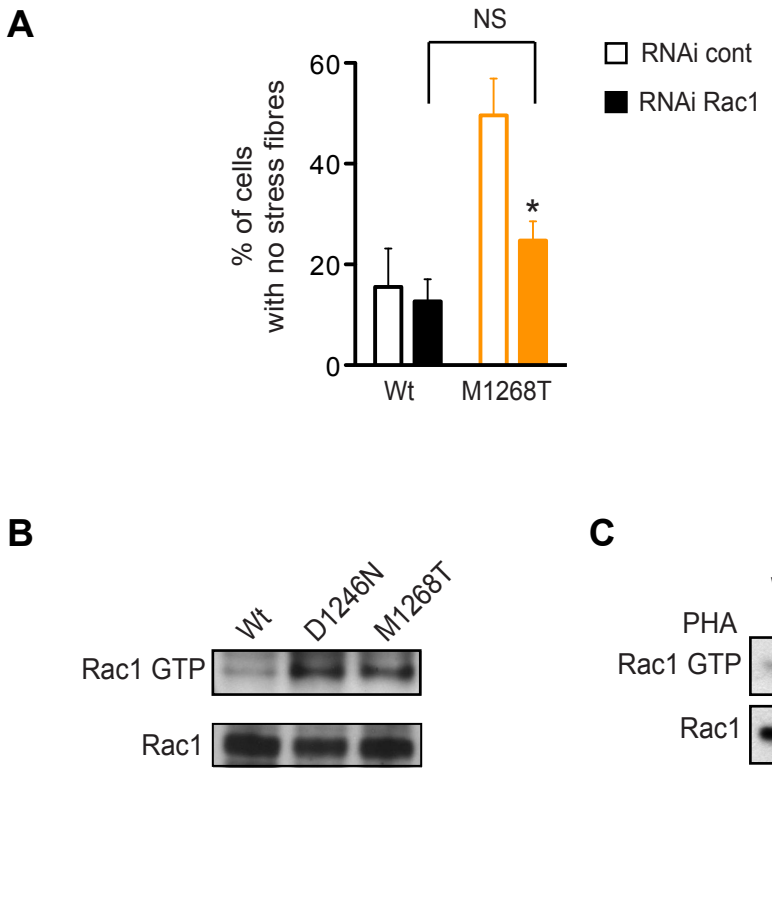
medium supplemented with 100μl FBS was added to each well after 5 hours. Experiments were performed or cells harvested after 72 hours.



Appendix Figure 1: Met is involved in breast cancer progression. Staining and analysis performed by Dr. Colan Ho-Yen
A-D) Immunohistochemistry staining of Met on human tissue sections. Pictures taken on (x40 objective). **A)** Section of ductal carcinoma *in situ* (DCIS). **B)** Section of invasive ductal carcinoma (IDC). **C)** Negative control: section of human pancreas, where no primary antibody was included. **D)** Positive control: section of human pancreas. **E)** Percentage of DCIS cases (n=47) and invasive cancer cases (n=153) with strong, intermediate and weak total (cytoplasmic and membranous) Met expression; $p<0.0001$.

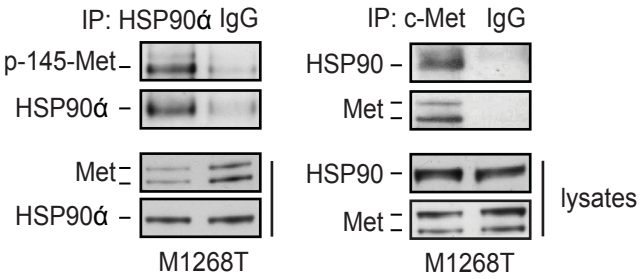


Appendix Figure 2: Met is present in endosomes in human tissue and increases as breast cancer progresses. Staining and analysis performed by Dr. James Hulit.
A) Immunofluorescence on human tissue sections of normal breast (800 cells)(top panel), DCIS (612 cells) (middle panel) and IDC (427 cells) (bottom panel) showing Met (red), EEA1 (green) and DAPI (blue).
B) Quantification of the percentage of early endosomes positively colocalising with Met (using Image J software). A minimum of 10 fields were analysed. Data are mean ± SEM. *=$p<0.05$.



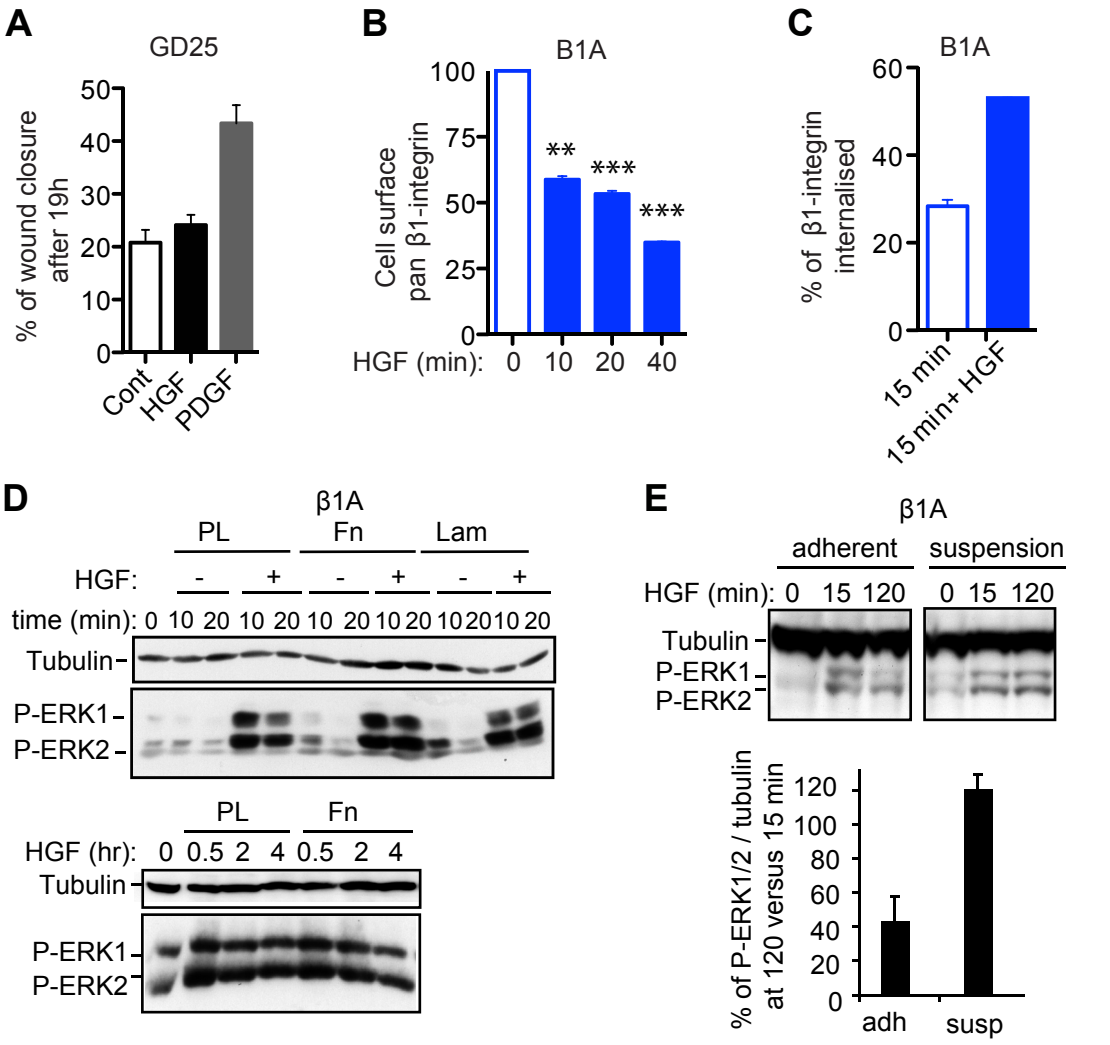
Appendix Figure 3: Carine Joffre showed that the D1246N and M1268T Met mutations induce Rac1 activation

A) Percentage of Wt and M1268T cells lacking stress fibres following transfection with control or Rac1 RNAi. Data are mean (arbitrary units) ± SEM (n=3). **B)** Levels of Rac1 GTP measured by GST-CRIB pull down and of total Rac1. **C)** Levels of Rac1 GTP measured by GST-CRIB pull down and of total Rac1 on Wt and M1268T expressing cells treated (+) or not (-) with PHA-665752. Numbers represent fold increases of Rac1 GTP between M1268T and Wt ± SEM (n=3). *p<0.05



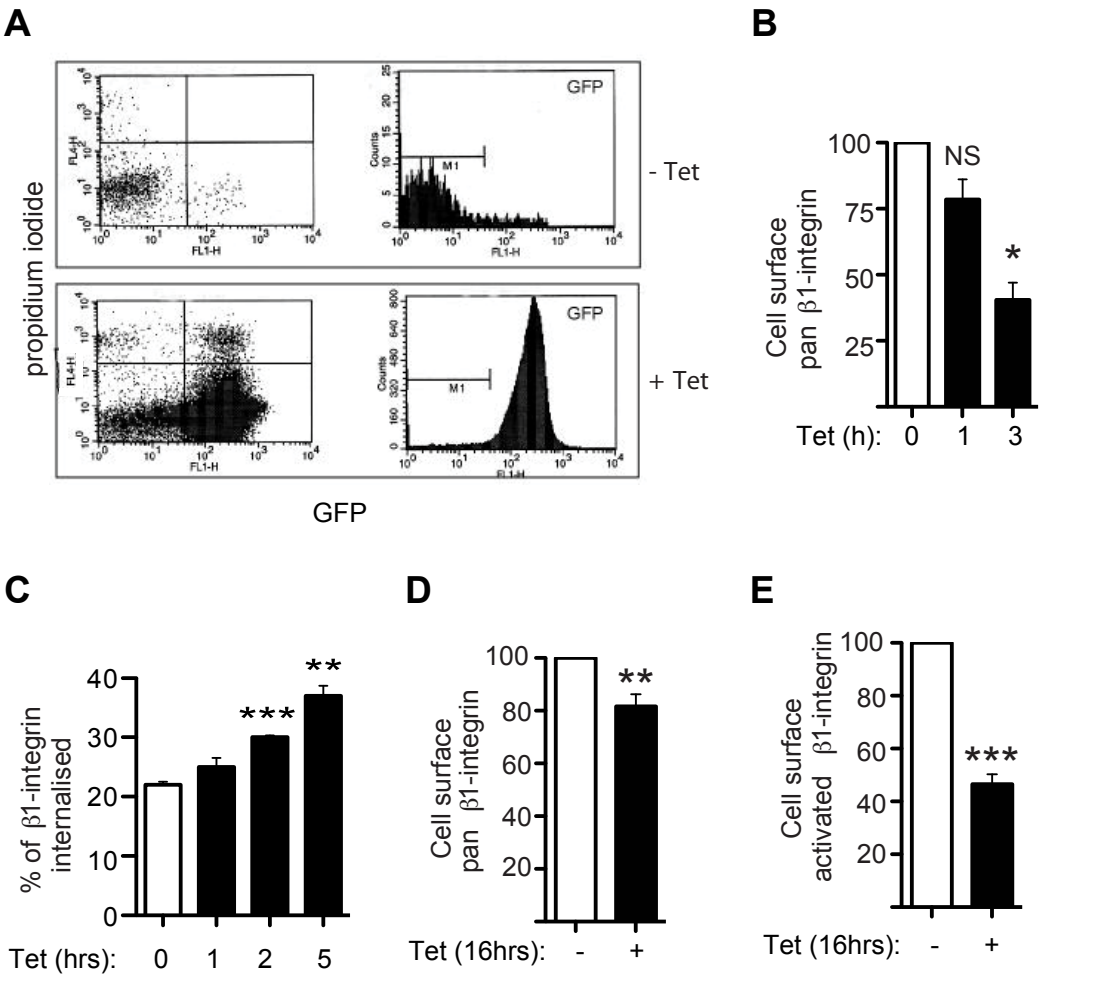
Appendix Figure 4: Carine Joffre showed that M1268T Met constitutively associates with HSP90 ά

Lysates from M1268T Met expressing cells were subjected to HSP90 ά IP (left panel) or Met IP (right panel). Cells were also subjected to an IgG control IP in each experiment. Met, HSP90 ά and pan HSP90 Western blot from IP or from total lysates are shown.



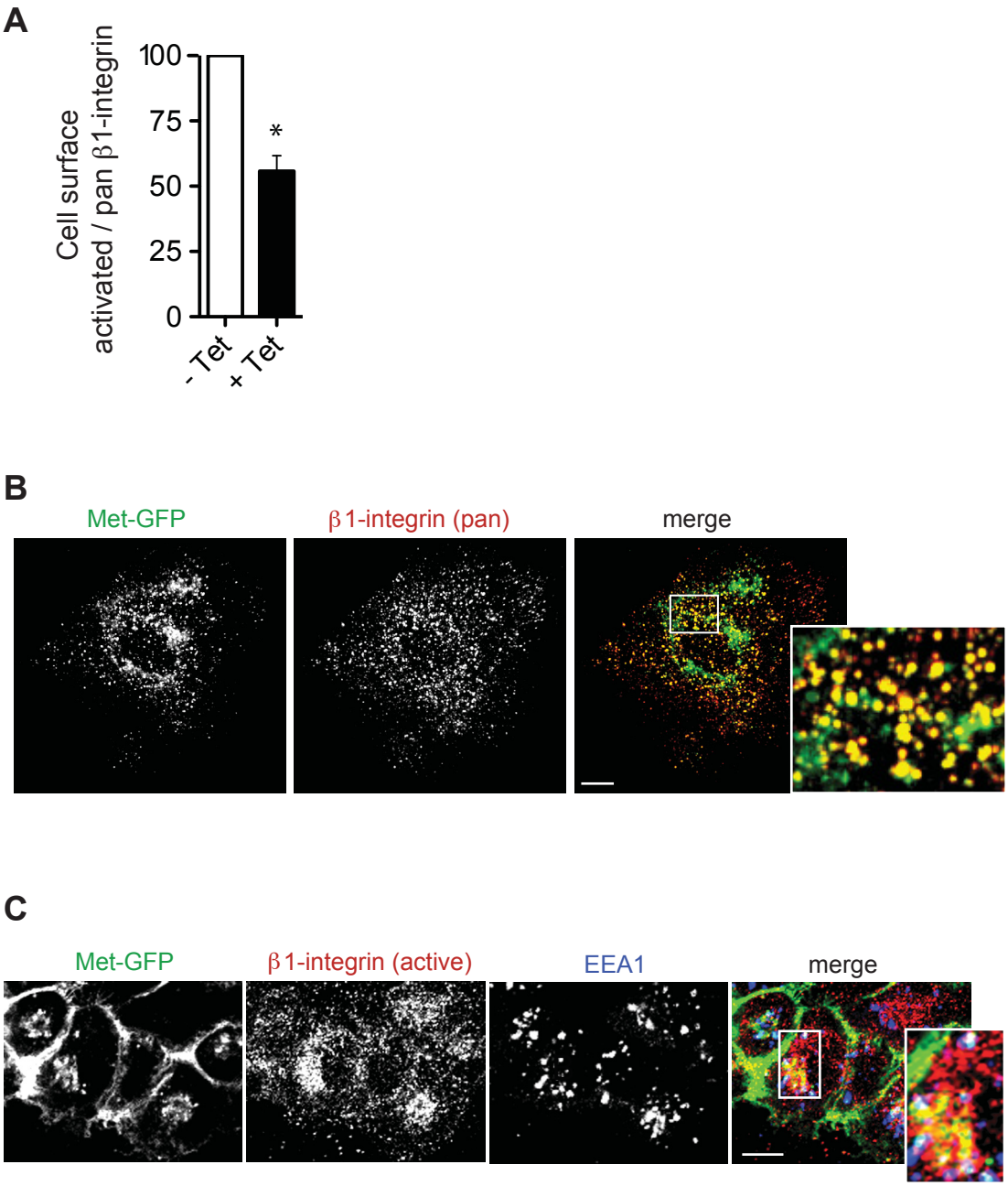
Appendix Figure 5: Met activation stimulates β1-integrin internalisation and is required for ERK1/2 phosphorylation in an anchorage independent manner.

A) Quantitation of wound closure from pictures (not shown) of GD25 cells non-stimulated, stimulated with HGF (50ng/ml) or PDGF (30ng/ml) for 19 hours. Data are mean (arbitrary units) ± SEM (n=1 in triplicate). Performed by Dr. Stephanie Kermorgant. **B)** Surface levels of pan- β1-integrin in β1A cells upon time of HGF stimulation, obtained by flow cytometry. Average mean fluorescence intensity, in arbitrary unit, ± SEM (n=3). Performed by Prof. J. Ivaska's laboratory. **C)** Biotinylation internalisation assay. In β1A cells, the levels of surface biotinylated β1-integrin internalised at 15 minutes, in absence or presence HGF, were measured and compared to total cell surface β1-integrin (mean ± range of error, n=2). Performed by Dr. Carine Joffre. **DE)** Performed by Dr. Stephanie Kermorgant. **D)** Western blots for tubulin and phospho-ERK1/2 in β1A cells non-plated (0) or plated on poly-L-lysine (PL). fibronectin (Fn) or laminin (Lam) for the indicated times without or with HGF. **E)** Western blots for tubulin and phospho-ERK1/2 in β1A cells, adherent or in suspension, and stimulated with HGF (50ng/ml) for 0, 15 and 120 minutes. Numbers represent the percentages of increase ± range of error (n=1 in triplicate), in phospho-ERK1/2/tubulin ratios at 120 versus 15 minutes. *p<0.05, **p<0.01,***p<0.001.

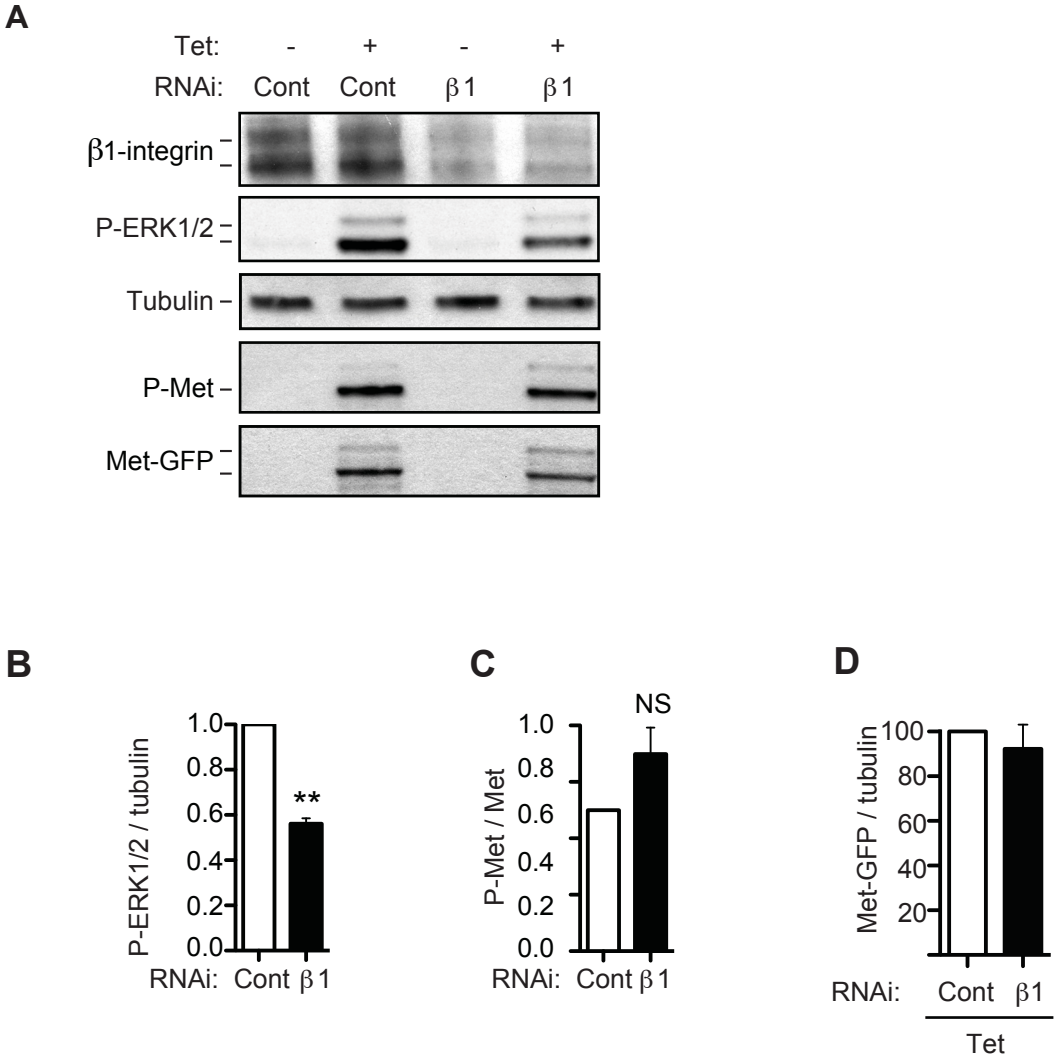


Appendix Figure 6: Expression of constitutively activated GFP-Met leads to β1-integrin internalisation.

A) GFP expression and viability analysed by flow cytometry. Met-GFP cells cultured in presence or absence of 0.1µg/ml of tetracycline (Tet) for 48hrs were stained with propidium iodide. Performed by Nick Luckens and Dr. Stephanie Kermorgant. **BC)** Performed by Prof. J. Ivaska's laboratory. **B)** Cell surface integrins of Met-GFP cells, induced with tetracycline (Tet, 0.1 µg/ml) for the times indicated, were labelled with anti-β1-integrin-antibody and analysed by flow cytometry. Data are mean ± SEM (n=3). **C)** Biotinylation internalisation assay, in which the amount of internalised β1-integrin within 30 minutes was evaluated in cells that have been induced with tetracycline (Tet) for the times indicated and compared to total cell surface β1-integrin (n=3). Data are means ± SEM. **DE)** Performed by Dr. Carine Joffre. Results of cell surface flow cytometry experiments performed with **D)** pan-β1 integrin (DF7) or **E)** activated conformation β1-integrin (9EG7) antibodies in cells non-treated (-Tet) and treated (+Tet) by tetracycline for 16 hours. Data are mean (arbitrary units) ± SEM (n=4). *p<0.05; **p<0.01; ***p<0.01; NS=not significant.



Appendix Figure 7: Met colocalises with β1-integrin on endosomes
A) Results of flow cytometry experiments performed with pan-β1-integrin (DF7) or activated conformation β1-integrin (9EG7) antibodies in Met-GFP cells non treated (-Tet) and treated (+Tet) by tetracycline for 16 hours. Data are cell surface active conformation β1-integrin expression reported on the cell surface pan-β1-integrin expression (arbitrary units) ± SEM (n=4). Performed by Dr. Carine Joffre. **B)** Confocal section of Met-GFP cells that have been induced with tetracycline for 5 hours and stained for β1-integrin (red). GFP-tagged Met is in green. Scale bar: 20μm. Performed by Prof. J. Ivaska's laboratory. **C)** Confocal section of Met-GFP cells, grown on Poly-L-lysine coated coverslips, fixed and stained for active β1-integrin (9EG7, red) and EEA1 (blue), after incubation in tetracycline for 16 hours. GFP-tagged Met is in green. Scale bar: 10μm. Performed by Dr. Stephanie Kermorgant.



Appendix Figure 8: β1-integrin is required for Met dependent ERK1/2 phosphoylation in an anchorage independent manner.
Experiments performed by Carine Joffre and Naoki Kishi
A) Western blots for β1-integrin, P-ERK1/2, tubulin, P-Met (Y1234/35) and GFP (Met-GFP: p195, precursor; p170, mature β chain) on Met-GFP cells, transfected with control or β1-integrin siRNA for 72 hours and incubated in tetracycline (Tet, 0.1 µg/ml) for 0 or 16 hours. **BC)** Fold increases ± SEM (n=3) of **B)** P-ERK1/2 / tubulin and **C)** P-Met/Met-GFP ratios in cells knocked down for β1-integrin versus control knocked down. **D)** Quantitation of Western blots obtained from Met-GFP cells, transfected with control or β1-integrin siRNA for 72 hours and incubated in tetracycline (Tet, 0.1 µg/ml) for 0 or 16 hours. Numbers represent relative levels (arbitrary units) ± SEM (n=3) of p170 Met-GFP / tubulin ratios. *p<0,05, **p<0.01, ***p<0,001

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